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ENZYMES OF POLYNUCLEOTIDE METABOLISM

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Part I. Crystalline Pancreatic Ribonuclease

INTRODUCTORY REMARKS

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A few remarks about the structure of bovine pancreatic ribonuclease, as it is now known, may be useful. Contributors to this part of the monograph will discuss material that can be considered in a rational way only by reference to the covalent structure of the enzyme. The present state of these investigations is illustrated by FIGURE 1, which has been constructed from the combined studies of a number of individuals at the Rockefeller Institute, New York, N. Y

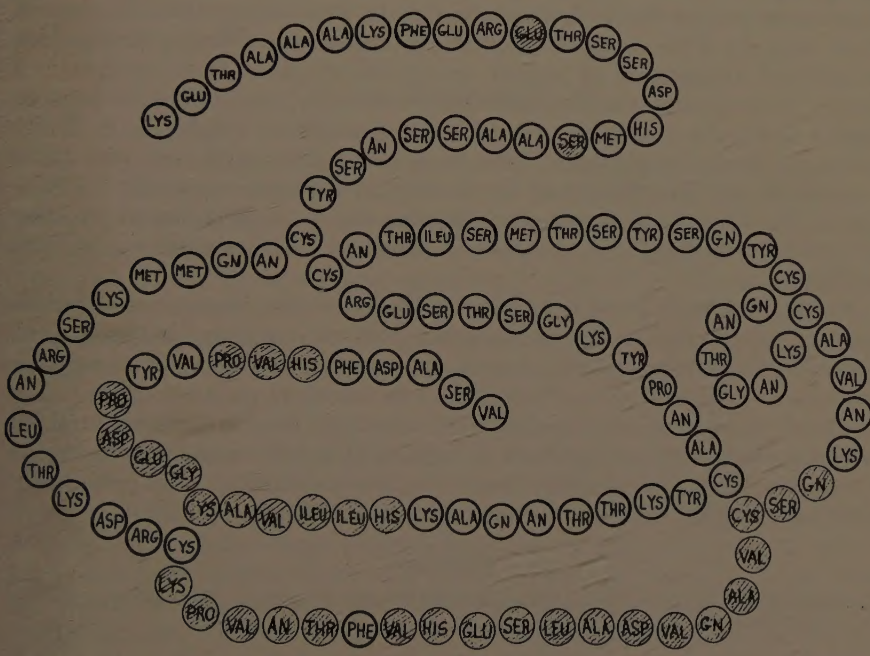

FIGURE 1. A partial structural formula for bovine pancreatic ribonuclease. The positions of the crosshatched residues are to be considered as arbitrary at present (see text).

(including C. H. W. Hirs, S. Moore, W. Stein, D. Spackman, and L. Bailey), and at the National Heart Institute (including R. Redfield, J. Cooke, A. Ryle, and myself). References to the various papers that describe the details of structural analysis are summarized, for the most part, in articles included in the recent book *Symposium on Protein Structure.*[1] The positions of the four disulfide bridges are probably as shown in the figure, although there is still some question regarding the bridges between the second and eighth and between the third and seventh half-cystine residues, because of the poor yields

obtained during the isolation of cystine peptides and because of the possibility of chemical rearrangements that might have occurred during certain steps in the sequence analysis of these peptides. At present, about 95 residues may be assigned with reasonable certainty to definite positions in the polypeptide chain, which contains 124 residues in all. In the figure those residues that are crosshatched may be assigned to the general region of the molecule indicated, but their exact position in the sequence must await further investigation by stepwise degradation. Hirs is in the process of completing these studies and it is hoped that the final results will be available in a few months. Our own group has not continued with the systematic study of sequence since the spring of 1956, and our independent contribution to the sequential structure consists, therefore, of approximately 45 residues, located for the most part in the N-terminal region of the molecule, in the region between the 1-6 and 2-8 disulfide bridges, at the C terminus, and around the 4-5 disulfide bridge. In the meantime we have been able to duplicate the results obtained by Hirs, Moore, and Stein in regard to the composition of the peptides produced during the digestion of oxidized ribonuclease by trypsin and chymotrypsin. These confirmatory results, which include some by-products of studies on species differences between bovine and ovine pancreatic ribonucleases (mentioned briefly by F. White elsewhere in this monograph), have been in complete agreement with those reported by the investigators at the Rockefeller Institute, except for the location of the glutamic acid and serine residues shown in the figure at positions 11 and 18, respectively, the positions of which are to be reversed on the basis of Hirs' data.

Much of this monograph is concerned with the relative importance of various parts of the structure to biological activity, both enzymatic and immunological. It becomes clear that certain parts of the structure appear to be superfluous and other parts essential, at least insofar as our assay methods tell us. The question of whether the "unessential" structures are really unessential, or whether these parts are involved in aspects of the intracellular behavior of ribonuclease that are too subtle for us to appreciate at the present time, might well make a splendid subject for discussion in the future.

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SOME RELATIONSHIPS OF STRUCTURE TO FUNCTION IN RIBONUCLEASE

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One of our basic objects in the study of ribonuclease (RNase) has been determination of the structural features essential for its enzymatic activity. The preliminary approach has involved a study of chemical modifications of the enzyme in relation to activity, with conclusions being drawn as to the parts of the molecule affected and the likelihood that these parts were associated with enzymatic activity. There are numerous reports of this kind in the literature on many enzymes; RNase is one of the more thoroughly studied in this respect. As indicated in TABLE 1, some treatments destroy RNase activity while others do not. It can be inferred from such results that only a part of the enzyme molecule (the "active center") is essential for catalysis.

Physical Studies on RNase

The changes in certain physicochemical properties have been studied to gain further insight into the nature of the chemical changes that accompany inactivation. The ultraviolet (U. V.) spectrum of acid hydrolyzates of RNase contains the expected peak for tyrosine with a maximum at 2760 Å. However, the corresponding peak in native RNase is displaced about 15 Å toward higher wave lengths and shows a higher maximum ($\epsilon = 9800$ for native RNase and 9390 for the hydrolyzate).[1-3] There is an abundance of evidence[4-7] that suggests that this effect is due to hydrogen bonds between tyrosine hydroxyl groups and carboxylate groupings. Most procedures examined thus far that cause the inactivation of RNase also eliminate this "native" spectral displacement. FIGURE 1 demonstrates the effects of various conditions on the U. V. spectrum of RNase. The displacement is abolished by limited pepsin digestion, the product of which apparently differs from native RNase only insofar as the C-terminal tetrapeptide is missing.[8] This product has little or no RNase activity. The displacement is also eliminated by 8 M urea, but the native U. V. spectrum is restored by addition of 0.15 M phosphate.[2] It has been suggested that restoration of the native spectrum by orthophosphate, polymetaphosphate, and certain other anions is duplicated by the substrate, ribonucleic acid (RNA), and that this effect accounts for the undiminished activity which RNase displays in 8 M urea despite elimination of the spectral displacement in the absence of these anions.[3, 9]

That it is possible to destroy enzymatic activity without abolishing the spectral shift was shown recently by Klee and Richards.[18] The 95 per cent guanidination of RNase resulted in a product with only 33 per cent of the specific activity of native RNase, but it exhibited only a lowering of the extinction, without the shift to a lower wave length.

F. M. Richards[10] has reported that limited digestion of RNase with the bacterial protease subtilisin yields an active product that consists of the N-terminal "tail" of twenty amino acids attached to the larger moiety, apparently

through hydrogen bonds. This product also exhibits the spectral displacement that is eliminated by urea and restored by phosphate. Separation of the N-terminal tail and the macromolecular component causes elimination of the native displacement that occurs together with complete loss of activity. Recombination of these components completely restores activity, as well as the spectral displacement. Neither part has the ability to regenerate activity on recombination after photo-oxidation. This reaction appears to affect only

TABLE 1
QUALITATIVE SUMMARY OF CHANGES IN ENZYMATIC ACTIVITY AND PHYSICAL PROPERTIES OF RIBONUCLEASE

Reaction	Changes from "native" properties			
	Activity destroyed*	Spectral shift**	Negative rotatory increase†	Viscosity increase†
Oxidation with performic acid[19]	yes	yes	yes	yes
Reduction to 1.5 SH[3]	slight	no	no	no
Reduction to 8 SH[3]	yes	yes	yes	yes
Limited pepsin digestion[2]	yes	yes	slight	slight
Complete pepsin digestion[2]	yes	yes	yes	–
Methylation[3]	yes	yes	yes	–
95 Per cent guanidination[18]	yes	no‡	no	–
8 M Urea[2, 30]	no	yes	yes	yes
8 M Urea + 0.15 M phosphate[2]	no	no	no	no
2 M Guanidine[3]	no	yes	yes	yes
2 M Guanidine + 0.15 M phosphate[3]	no	no	no	no
6 M Guanidine[3, 9]	yes	yes	yes	yes
6 M Guanidine + 0.15 M phosphate[3, 9]	yes	yes	yes	yes
$pH > 12.7$[2]	yes	yes	–	–
$pH < 2$[1, 2]	no	yes§	–	–
100° C. for 2 hours[2]	yes	yes	–	–
Liquid NH_3[2]	no	no	–	–
Aqueous NH_3 (28 per cent)[2]	no	no	–	–

* Complete or appreciable destruction, unless otherwise indicated.
** Shift of ultraviolet spectrum of native ribonuclease to that of the total acid hydrolyzate, whereby the maximum of the "tyrosine" peak moves 15 Å toward the ultraviolet and decreases in extinction.
† Taken as indication of unfolding of secondary structure.
‡ No shift in maximum, but decrease in extinction.
§ This shift is reversible; when the pH is increased for the purpose of enzyme assay, the "native" spectrum is restored, and the product exhibits full activity.

histidine, which has been postulated frequently to be part of the active centers of certain esterases and proteases;[11-17] therefore the N-terminal tail may play a direct role in the active center.

Occurring with the denaturation of proteins is an unfolding of the secondary structure, which results in increases in negative optical rotation and viscosity. It is noteworthy that limited pepsin digestion of RNase, while it abolished activity and the native spectral displacement, had little effect on rotation.[2] It has been concluded that the secondary structure of RNase under these conditions of digestion remains nearly intact, but that essentially only the tertiary structure necessary for activity has been disrupted.

In contrast to the effect of limited pepsin digestion, cleavage of the disulfide

bonds of RNase (whether by oxidation with performic acid[19] or by reduction with sulfhydryl-containing reagents)[20, 21] causes marked increases in the negative optical rotation and in viscosity, in addition to abolishing the native spectral displacement. These effects constitute evidence that cleavage of disulfide bonds causes disorientation of the secondary structure, in addition to disrupting the tertiary configuration.

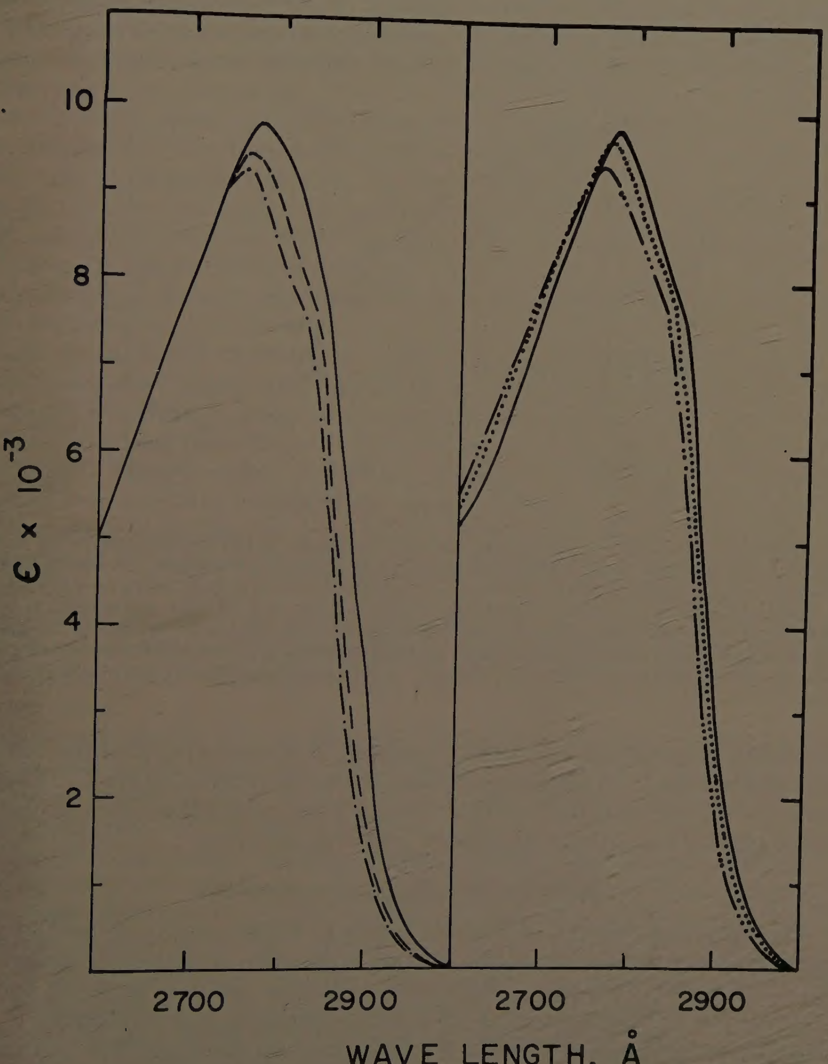

FIGURE 1. Curves represent absorption spectra as follows: —, native RNase in water, phosphate buffer, or a solution of 8 M urea and 0.15 M phosphate buffer of *p*H 6.5; – – – –, pepsin-inactivated RNase in water; —·—·, RNase or pepsin-inactivated RNase at *p*H 6.5 after complete digestion with pepsin at *p*H 1.8; —···—, RNase in 8 M urea; ····, RNase in 8 M urea and 0.003 M phosphate buffer of *p*H 6.5.

For all reactions of RNase thus far studied, experimental conditions that produced increase in the negative optical rotation also resulted in an increase in viscosity. Another physical property, rotatory dispersion, has not been studied as thoroughly, but has served as additional confirmation of the uncoiling of the secondary structure of RNase in urea and guanidine.[3] In every situation where physical properties were affected in such a manner as to indicate secondary unfolding, there was also an elimination of the native spectral displacement and a destruction of activity. In all cases, except that in which RNase was dissolved in 8 M urea, activity was lessened or destroyed by a treatment that modified the native spectral displacement, although changes in

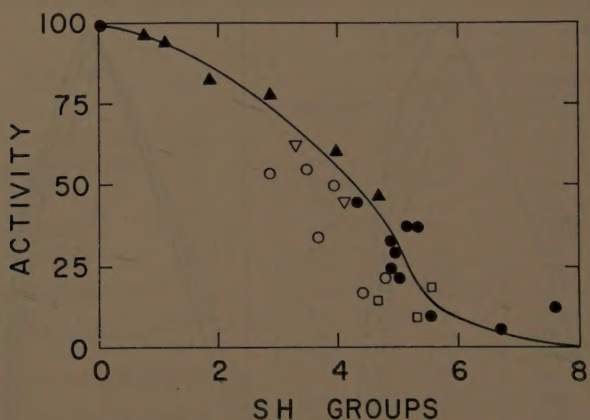

FIGURE 2. Enzyme activity (expressed as percentages of the specific activity of native RNase) as a function of the number of moles of SH per mole of enzyme. A *closed triangle* represents reduction in absence of urea; a *closed circle*, reduction in 8 M urea; an *open square*, reoxidation of RNase that had been reduced for 4.5 hours in 8 M urea and contained 8 SH groups per mole; an *open circle*, reoxidation of samples containing more than 6 SH groups per mole; an *open triangle*, reoxidation of samples containing about 4 SH groups per mole. The curve is drawn through the black points to indicate approximately the course of reduction.

secondary structure, as indicated by rotatory or viscosimetric changes, did not necessarily follow. A summary of these studies is given in TABLE 1. Experimental results thus far indicate that the active center of RNase involves folding of the peptide chain to create a specific tertiary structure.

Reduction and Reoxidation of RNase

The reductive cleavage of disulfide bonds in RNase results in loss of enzymatic activity, and a study of this reaction is therefore of interest in the investigation of the active center. Reduction of RNase has been achieved in this laboratory with thioglycolate at *p*H 8.5 (adjusted with trimethylamine) in the presence or absence of urea; further details are given elsewhere.[20, 21] FIGURE 2 summarizes a number of experiments undertaken to correlate enzymatic activity with sulfhydryl (SH) groups per mole of protein appearing on reduction of native RNase and reoxidation of reduced RNase; the curve is drawn through the black points to indicate the path of reduction, and the remaining points

represent some early experiments performed on the reoxidation of reduced RNase. It is noteworthy that these latter points tend to follow the same curve. It may be seen that inactivation proceeds at a relatively slow rate to about the half-reduced level, and then increases markedly. Thus it appears that the 2 disulfide bonds initially cleaved have little effect on enzymatic activity, while the final 2 are more intimately associated with the active center.

The locations of the disulfide bonds have been virtually established* by Spackman *et al.*[22] and Anfinsen and Ryle.[23] They appear to connect half-cystine residues 1–6, 2–8, 3–7, and 4–5. It has been reported[24] that the 1–6 bond is somewhat more labile than the others under the conditions of reduction employed in our laboratory. Since it appears to be absent at a level of reduction where considerable RNase activity remains, it has been concluded tentatively that this disulfide bond is not associated with the active center. However, the possibility that the function of disulfide bonds in catalysis is an entirely indirect one cannot be eliminated at present. It is possible that any disulfide bond, if cleaved while the others remained intact, similarly would be found to be unessential for activity.

According to Kern,[25] reduction of two of the three disulfide bonds in pepsin yields a partially active product that continues to lose activity with no further change in the extent of reduction. This observation was interpreted to mean that a secondary change was responsible for at least some of the activity loss, and that the absence of two of the three disulfide bonds permitted eventual denaturation and inactivation. These results would suggest that the function of disulfide bonds in pepsin is at least partially protective in nature. Similar results, however, have not been observed with RNase. The disulfide bonds in RNase seem more directly concerned with the active center than those in pepsin, and the question may be raised as to whether these bonds in RNase form a part of the essential structure of the active center or function in a protective manner. Upon reduction of trypsin, Liener,[26] found a complete loss of enzymatic activity with disappearance of only one of its three disulfide bonds. Here, also, a close connection is indicated between disulfide bonds and the active center.

Enzymatic activity lost on reduction of RNase may be regenerated by oxidation with molecular oxygen. Such reoxidation has yielded complete reactivation of reduced RNase, starting with samples having specific activities of 5 to 10 per cent that of native RNase.

Methods. Reduction and determination of the resulting SH content by spectrophotometric titration with *p*-chloromercuribenzoate were carried out as previously described.[20, 21] The reoxidation procedure involves aeration of solutions of reduced RNase. Phosphate, citrate, and barbital buffers have been used for maintenance of *p*H, with no significant differences noted in the extent of reoxidation or reactivation. Results of a preliminary study of the effects of *p*H on activity and SH content are shown in FIGURE 3. The disappearance of SH groups was markedly less rapid at low *p*H values, and the *p*H range for maximum regeneration of activity was quite broad, ranging from 6.1

* Because of the possibility of some chemical rearrangement during isolation and characterization of cysteic acid peptides, some uncertainty remains in regard to the 2-8 and 3-7 bonds.

to 8.6. A tendency toward precipitation of protein was noted in some experiments, which possibly was the result of intermolecular coupling of SH groups to form polymers. Precipitation generally was minimized by reducing the rate of aeration to 1 bubble every 5 sec. At *p*H 8.6, precipitation was more marked, hence such a high *p*H was considered undesirable. The *p*H range for oxidation was limited further by the solubility of reduced RNase. At advanced levels of reduction (higher than 7 SH groups per mole) RNase is only slightly soluble between *p*H 6 and 7. Therefore most of the oxidation studies were performed between *p*H 7 and 8, with 0.01 M sodium phosphate buffer. The protein concentration was routinely 1 mg./ml. Reoxidized RNase was pre-

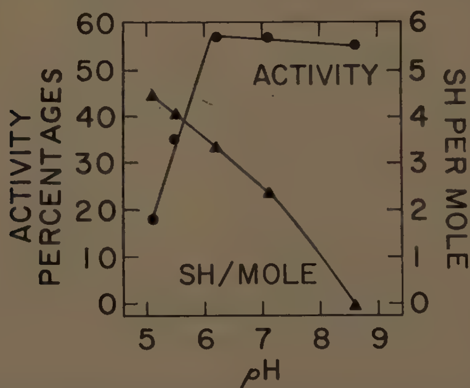

FIGURE 3. SH groups per mole of protein, and enzyme activity (expressed as percentages of the specific activity of native RNase) on reoxidation of reduced ribonuclease as functions of *p*H. Enzyme activities were determined using ribonucleic acid as the substrate.[19] A *closed circle* represents variation of activity with *p*H; a *closed triangle*, variation of SH groups per mole of protein according to *p*H. The RNase had been reduced for 3 hours in 8 M urea as previously described[20, 21] and possessed 7.5 SH groups per mole (by titration with *p*-chloromercuribenzoate) at the start of reoxidation, which was carried out for 68 hours as described in the text. The *p*H values were maintained throughout oxidation by 0.01 M phosphate buffers.

pared for physical studies by dialysis against distilled water and by lyophilization.

Results and discussion. FIGURES 4 and 5 summarize oxidation studies on RNase at 3 levels of reduction. Insofar as we have been able to ascertain, there is no difference between the levels of reduction of RNase reduced for 4.5 hours and that reduced for 24 hours, both reactions being carried out in 8 M urea. However, the results on oxidation of RNase that had been reduced for 24 hours indicated much less elimination of SH and regeneration of activity than for material reduced only 4.5 hours. A possible explanation is that the protein structure is further modified, nonspecifically and irreversibly, after all of the disulfide bonds have been broken. As controls, 4.5-hour-reduced carboxymethylated RNase and native RNase were oxidized in the same manner; activity, however, was not altered by this procedure.

Preliminary physical studies have been conducted with RNase that had been reduced for 60 min. in 8 M urea to yield 6.6 SH groups per mole (activity, 5 to

10 per cent of native RNase), and then reoxidized for 40 hours. Enzymatic activity was completely regenerated by this procedure. A decrease in negative rotation resulted from reoxidation; this finding is consistent with the conclusion

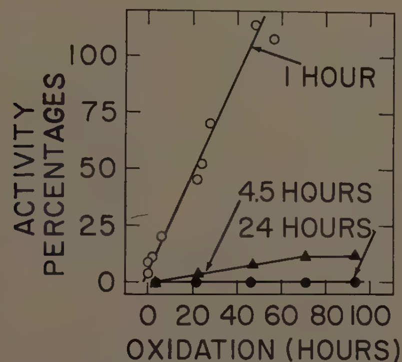

FIGURE 4. Changes in enzyme activity (expressed as percentages of the specific activity of native RNase during reoxidation of reduced RNase. Enzyme activities were determined using ribonucleic acid as the substrate.[19] All protein samples had been reduced in 8 M urea prior to oxidation, as previously described.[20, 21] An *open circle* represents RNase reduced 1 hour; a *closed triangle*, RNase reduced 4.5 hours; a *closed circle*, RNase reduced 24 hours. The *p*H of the reaction mixtures was maintained at 8 with 0.01 M phosphate buffer.

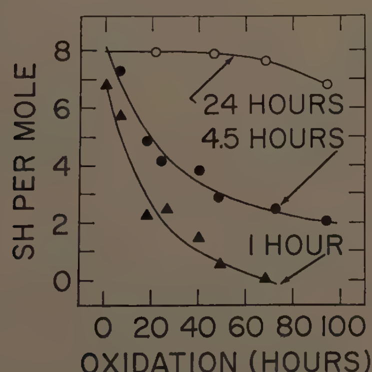

FIGURE 5. Changes in SH groups per mole of protein during reoxidation of reduced RNase. SH groups were determined by spectrophotometric titration with *p*-chloromercuribenzoate.[21, 31] All protein samples had been reduced in 8 M urea, as previously described,[20, 21] prior to oxidation. A *closed triangle* represents RNase reduced 1 hour; a *closed circle*, RNase reduced 4.5 hours; an *open circle*, RNase reduced 24 hours. The *p*H of the reaction mixtures was maintained at 8 with 0.01 M phosphate buffer.

that the reoxidized product possesses at least as much helical coiling as the native molecule. Further support for this conclusion comes from the observation that viscosities of the native and reoxidized enzymes are similar, and that both are significantly lower than that of the 60-min.-reduced carboxymethylated RNase. Finally, as a result of reoxidation, there is a nearly complete return to the U. V. spectrum of native RNase. These preliminary studies strongly suggest a re-formation of secondary and tertiary structure that, al-

though possibly not identical to that of native RNase, is sufficiently similar to it to permit complete regeneration of enzymatic activity.

Species Differences

Investigation of species differences in molecular structure of RNase may be expected to yield some valuable information on relationships between structure and function in this enzyme. Such work was initiated in our laboratory by Stig Åqvist.[28, 29] Serine is substituted for threonine at position 3, and glutamic acid for lysine at position 37 in ovine pancreatic RNase as compared to the enzyme from beef pancreas. In addition to these variations, there is evidence for at least 1 more difference, but the details must await further investigation. It should be noted that Residue 3 represents a difference of only 1 methylene group, which might be insufficient to produce a difference in specific activity even if this residue were involved directly in the active center. Aside from this precaution, the inference may be made that, since these positions are interchangeable from beef to sheep without change in specific activity, they are not involved in the active center. Work is in progress in our laboratory (under the direction of Trygve Tuve) on the purification of spinach RNase. Further comparative structural studies are planned with this enzyme and with porcine ribonuclease, which is now available in pure form.

Summary

RNase activity depends upon a variety of structural features. Some of these may be functionally involved in the binding of substrate, and others may form a structural part of the active center or may act as determinants of its proper 3-dimensional orientation. It would appear that at least 2 histidine residues, 1 of which is located in the N-terminal tail of 20 amino acids, participate directly in the process of catalysis. From the evidence at hand, hydrogen bonds between tyrosine hydroxyl groups and unidentified carboxylate groups play a role in the tertiary structure of the active center. There is evidence that 1 lysine residue[18] is essential for RNase activity.

On the basis of studies on the reduction of disulfide bonds, certain of them appear to be obligatory for activity. The importance of disulfide bonds to activity is further substantiated by the reappearance of activity upon reoxidation of the reduced enzyme. Preliminary physical studies on the reoxidized product suggest a refolding to a structure similar to that of the native enzyme.

Acknowledgment

We thank W. F. Harrington, who performed some of the preliminary physical studies on reoxidized RNase.

Addendum

It has been observed[31] that thiolesters, present as impurities in thioglycolic acid, may undergo a "thiolation" reaction[32] with protein amino groups during reduction. This difficulty can be eliminated by vacuum distillation of thioglycolic acid before it is used in reduction. Recent unpublished results on reduction and reoxidation of RNase indicate that thiolation may account in large part for the nonspecific alteration (discussed above) of RNase during reduction. RNase was devoid of enzymatic activity when treated under conditions similar to those described[20, 21] for complete reduction (except that vacuum-distilled thioglycolic acid was used as the reducing agent). However, when this protein was reoxidized by the methods

given above, values for regenerated RNase activity were observed to approach those of the native enzyme.

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IMMUNOCHEMICAL REACTIONS INVOLVING RIBONUCLEASE*

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Much is known about the amino acid sequence and disulfide bonds[1, 2] of bovine pancreatic ribonuclease (RNase). Its demonstrated antigenicity[3] has prompted study of the structural features necessary for its reaction with antibody. The immunologically reactive portions of many polysaccharide antigens have already been studied[4] and, in the case of blood-group substance,[5] the approximate size of the reactive portion is known.

This paper summarizes chemical and enzymic experiments that have delineated some of the groups concerned with the reaction between RNase and its antibody.

Production and Characterization of Antibody

Rabbit antibody to ribonuclease (Ra-a-RNase) was prepared by intravenous injection of RNase or chromatographically purified RNase *A* in increasing doses of alum precipitate, either daily or every other day. A total dosage of about 50 mg. was used and the animal was bled 6 days after the last injection. The antibody was tested for the presence of contaminating immune systems by diffusion into an agar-antibody mixture by the method of Oudin[6] and by simultaneous diffusion of antigen and antibody from wells on an agar plate, according to the procedure of Ouchterlony.[7] All sera used showed only a single line of antigen-antibody reaction, indicating immunological homogeneity.

Tests of the supernatant solutions from specific precipitates provided another index of immunological purity. One aliquot of each supernatant solution was tested with a small amount of antigen and another with a small amount of antibody. Precipitation should not occur in both tests if a single immune system of low solubility is present; no sera used showed contaminating immune systems by this method. Electrophoresis in agar and subsequent diffusion of antibody into the path produced a single line of immune precipitate;[8] this is another criterion of a single immune system. These tests were done with RNase that had not been chromatographed, since the antigen contaminants might have been removed, and the corresponding antibodies would then have remained undetected. In some tests, chromatographic fractions rich in contaminants were used. None of the sera used showed evidence of contaminating immune systems by any of these tests. All of the tests merely exclude contaminants; no procedures are available to test purity directly.

Once it is established that a single immune system is present, it is necessary to identify it. This was done by testing the washed specific precipitates for enzyme activity. The antibody was destroyed by urea denaturation before the assays were done. Ninety to 95 per cent of the added RNase activity[9] was recovered in the washed specific precipitates. The following controls indicate that this RNase was precipitated by specific interaction. When

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RNase *or* Ra-a-RNase was added to the rabbit antihuman serum albumin–human serum albumin system, *no* RNase was recovered in the urea-treated precipitate. Further evidence that the antibody was directed toward RNase was obtained by overlaying 1 per cent agar, containing 13 μg. Ra-a-RNase N per milliliter, with various concentrations of RNase. The rate of downward movement of the line of precipitation was measured with a cathetometer and used to calculate the diffusion constant ($D_{20,w}$) of the antigen. The $D_{20,w}$ of 9.1×10^{-7} cm.^{2}/sec. is in good agreement with the value of 9.9×10^{-7} cm.^{2}/sec. from the literature.[9] In addition to providing further identification of the immune system, this $D_{20,w}$ indicates that the RNase does not behave as a haptene, bound perhaps to some serum protein, but as a free complete antigen.

RNase A and B

Crystalline RNase is chromatographically inhomogeneous, consisting of at least 2 small fractions that appear early during the chromatographic run and are followed by a larger component, RNase *B*, and a final major peak, RNase *A*.[10] The minor components did not react with antibody. RNase *A* and RNase *B* were indistinguishable in their reactivity toward Ra-a-RNase. By double diffusion in agar, a single line of identity was obtained. Either RNase *A* or RNase *B* removed all of the antibody to both. Increasing amounts of each of the components were added to separate aliquots of antiserum, and portions of the supernatant solutions were tested with small amounts of each antigen. In no case was antibody detected with 1 antigen and not the other. Economy of antigen and antibody can be achieved by studying their interaction in very dilute systems and by estimating the amount, $C'H_{50}$, of guinea pig complement that they destroy or fix. A mixture of sheep cells and antibody to them is used as an indicator of residual complement.[11] Aliquots of each chromatographic fraction were analyzed for RNase by C′ fixation, by the ninhydrin reaction, and by enzyme assay. Good agreement was obtained by all 3 methods. Thus, RNase *A* and *B* are immunologically indistinguishable. These findings are in agreement with the data of Cinader and Pearce.[11a] RNase *A* and *B* probably differ only in that 1 carboxyl group of RNase *B* is replaced by an amide group in RNase *A*.[12]

Hydrogen Bonds

The change in physical properties and in the kinetics of deuterium oxide exchange[13] suggest that many of the hydrogen bonds of RNase are disrupted in concentrated solutions of urea or guanidine. Unfortunately, antibody is destroyed irreversibly under these conditions, so it is not possible to determine if certain of these bonds are required for immune reactivity. It is probable that all of these bonds re-form upon removal of the urea or guanidine. Nevertheless, experiments were done to see if such procedures altered the immune reactivity of RNase. RNase was dissolved in concentrated neutral solutions of urea or guanidine and then separated by dialysis or precipitation with alcohol. Another RNase solution was heated in boiling water. Neither of these treatments affected the reaction of antibody with RNase. Ethanol, dioxane,

diethyl ether, or aqueous solutions of these substances do not affect RNase irreversibly, but ethylene chlorhydrin and tetrahydrofuran destroy its immunological reactivity. This action probably involves more than destruction of certain nontyrosyl hydrogen bonds. If any hydrogen bonds are irreversibly destroyed by boiling or by urea or guanidine treatment, they are not essential for immune reactivity.

The hydroxyl groups of 3 tyrosyl residues of RNase titrate abnormally and irreversibly in aqueous solution.[14] Again, the instability of Ra-a-RNase prevents studies at alkaline *p*H, and only the essentiality of the irreversibly altered structures can be tested. RNase was brought to *p*H 12.7 and examined spectrophotometrically. After the optical density at 294 mμ became constant, the RNase was titrated to neutrality and examined for chemical alteration. A 10 per cent increase in ninhydrin color occurred, but was also present in control samples brought to a *p*H of only 11.5. No new N-terminal amino acid residues were found by analysis with fluorodinitrobenzene (FDNB), and only a small amount (0.12 mole per mole) of SH groups appeared. When tested by C′ fixation, much larger amounts of alkaline-denatured RNase were required to reach maximal fixation, and the fixation (amount of C′ destroyed in $C'H_{50}$) was smaller.

Alkaline-denatured RNase was studied further by chromatography on IRC-50 at *p*H 6.18. Two major and 3 minor peaks appeared. Each major component fixed as much C′ as native RNase but, compared with native RNase, 2000 times as much was required of one and 200 times as much of the other for equivalent fixation. This was not due solely to the presence of a small amount of unchanged RNase, since each fraction inhibited the RNase-a-RNase reaction much more strongly than could be accounted for by the amount of unaltered RNase the fraction could have contained, namely, 0.05 per cent and 0.5 per cent, respectively.

Recently, it has been shown that the hydroxyl groups of all six tyrosyl residues can be titrated reversibly in certain solvents, and that the loss in enzymic activity by alkaline denaturation is secondary to the tyrosyl-bond disruption.[15] Thus, whatever occurs following tyrosyl-bond rupture probably alters the immune reactivity of RNase.

The ubiquity of hydrogen bonds suggests that some will be broken during most chemical alterations; it must be borne in mind that their rupture, rather than the breakage of covalent bonds, may be responsible for any change in immune reactivity.

Disulfide Bonds

The four disulfide bonds of RNase may be destroyed by oxidation with performic acid or by reduction with reagents containing an SH group. RNase that had been treated with performic acid did not react with Ra-a-RNase by agar diffusion or by C′ fixation and did not inhibit the reaction of RNase with Ra-a-RNase. Since alteration of the methionine and cysteine residues occurs with this treatment, reduction with thioacetic acid, thioglycol, and 2,3-dimercaptopropanol (BAL) was tried. The enzymic and immunological activities of RNase were lost at about the same rate. No evidence of appreciable amounts of partially active intermediates was found under these conditions,

although such a substance (or substances) was prepared by altering the conditions.

It has been suggested that a certain disulfide bond, or bonds, may be unessential for the enzymic activity of RNase, and that this bond may be destroyed preferentially by treatment with thioglycollate at *p*H 8.5 in water.[16] Under these conditions the extent of reduction as measured by the Boyer procedure[17] varied in different experiments, although in each the extents of enzymic and immunological destruction were similar. If iodoacetic acid was added immediately after reduction (to prevent reoxidation of the SH residues), a derivative was obtained that gave peak fixation at concentrations comparable to

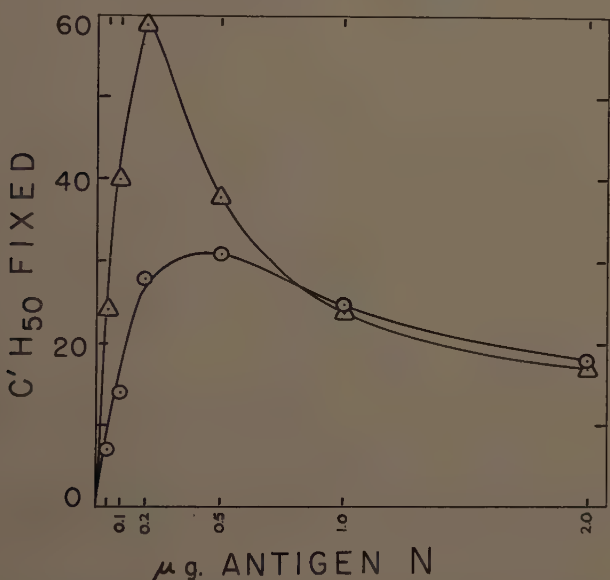

FIGURE 1. Complement fixation with native RNase (*triangle*) and partially reduced RNase blocked by iodoacetic acid (*circle*).

native RNase, but that fixed appreciably less C′ (FIGURE 1). Iodoacetic acid added to native RNase as a control did not affect the immune reactivity of the RNase. The enzymic activity was 5 per cent of the initial activity. The extent of reduction of this derivative was not calculated, but a similar unblocked preparation had 4.0 SH groups per mole. It is not possible to determine whether this derivative represents a mixture of products from random reduction of disulfide bridges or if certain bonds are reduced more readily. The partial reactivity of the derivative cannot be due to a mixture of inert and completely reactive RNase, since this would fix as much C′ as native RNase, although at higher antigen concentration. The derivative must contain an inhibitory product or a partially reactive product whether inert or unaltered materials are present. Thus, 2 or more disulfide bonds are essential for the immune reactivity, but 1 or more of the other bonds may be destroyed without complete loss of reactivity.

Peptide Bonds

Studies of the derivatives of RNase formed by proteolytic enzymes afford an opportunity to examine certain specific portions of the molecule, as well as the general class of peptide bonds. The immune reactivity of RNase, as judged by C′ fixation, was completely destroyed by digestion with pepsin or chymotrypsin. The rates of loss of enzyme and immune reactivity were similar. Thus, 1 or more of the bonds broken by each of the effective proteinases must be needed for immunological reactivity. Trypsin, thrombin,[18] spleen extract,[19] and cathepsin C did not break any peptide bonds of native RNase. Although prolonged pepsin treatment destroys RNase activity, a derivative may be prepared that is deficient in the C-terminal tetrapeptide only.[19a] This material reacted poorly with Ra-a-RNase, fixing less C′ than native RNase (TABLE 1).

TABLE 1

C′ FIXATION WITH FRAGMENT FROM PEPSIN-DIGESTED RNASE

Experiment No.	Antigen N, μg.	$C'H_{50}$ Fixed
I*	Native RNase	
	0.04	21
	0.08	39
	0.16	75
	0.32	81
II*	Digestion fragment	
	0.05	10
	0.50	31
	1.00	44
	2.00	41
	4.00	31
	8.00	21

* One hundred and ten $C'H_{50}$ were present in Experiment I and 115 $C'H_{50}$ in Experiment II.

Partial digests with α-chymotrypsin were prepared as follows: RNase *A* was digested with one tenth its weight of chymotrypsin at *p*H 7.3 in 0.05 M phosphate buffer for 44 hours at 40° C. The fraction dialyzable through $1\frac{8}{32}$ Visking tubing had lost all enzymic activity, did not precipitate with Ra-a-RNase, and did not inhibit the RNase-a-RNase reaction. The nondialyzable fraction retained 50 per cent of its enzymic and immunological activity.

Immunoelectrophoresis in agar done as described by Grabar and Williams,[8] but with glycine buffer at *p*H 9.5 to avoid the interaction retention of the RNase with the agar, gave a single line of precipitation for both native RNase and the nondialyzable fraction. The electrophoretic mobility, measured by the maximum of the arc of precipitation, was about the same. This was verified by electrophoresis in agar with staining after the 4 hours of migration according to Uriel's technique.[20] Ten times as much sample was used for staining as was used for immunoelectrophoresis. The nondialyzable fraction had a major component, whose electrophoretic mobility was near that of native RNase, and a small amount of 2 other more rapidly migrating components.

Double diffusion in agar against Ra-a-RNase, according to the Ouchterlony

technique, gave a reaction of identity between the native RNase and the nondialyzable fraction.

Chromatography on IRC-50, at *p*H 6.15, in 0.2 M phosphate buffer, separated the nondialyzable fraction into 2 major components and 3 intermediate fractions. The first peak chromatographed near a hold-up volume and had no immunological or enzymic activity. The minor components each had a diminished enzymatic activity compared to RNase, and their complement fixation curves had the same maxima which, however, were reached with different concentrations of antigen. The second major component appeared to be unaltered RNase, as judged by chromatographic, immune, and enzymic behavior, and by N- and C-terminal amino acid content. Thus, no appreciable amounts of active chymotrypsin fragments were obtained by this procedure.

Carboxypeptidase *A* will remove the C-terminal valine from intact RNase, and this has been shown to be nonessential for enzymic activity. Digestion of a 1 per cent solution of RNase for 22 hours with diisopropyl fluorophosphate-treated carboxypeptidase *A* gave 0.30 moles of valine per mole by the FDNB procedure. No change occurred in immune reactivity. Thus, this valine residue does not seem essential for immune reactivity. These results, coupled with the data from experiments with the pepsin fragment, suggest that all or a portion of the following residues or adjacent bonds near the C-terminal end of RNase must be intact before RNase can react with its antibody: aspartic acid (121), alanine (122), and serine (123).*

N-terminal Region

Attempts were made to digest the N-terminal amino acids from RNase with leucine amino peptidase (LAP) by mixing 9 mg. of RNase with 1 mg. of LAP ($C_1 = 60$) in 0.005 M Tris buffer containing an optimal magnesium concentration. No appreciable increase in ninhydrin color occurred during 12 hours at 40° C. Because the N-terminal region of RNase was resistant to LAP, chemical derivatives were the choice for study. Several different procedures were used wherever possible to help rule out the possibility of side reactions. RNase was deaminated by mixing a 2 per cent solution in molar, *p*H 4.0 acetate buffer with 1 volume of freshly prepared 2 M sodium nitrate at 0° C. The reaction was stopped by adding 20 volumes of absolute ethanol. Immune reactivity dropped quickly during the first 3 min., but was constant during the next 27 min. Thereafter, it gradually decreased until the termination of the experiment in 19 hours. RNase treated for 7½ min. was chromatographed on IRC-50 at *p*H 6.10. Two major peaks were found. By Van Slyke analysis, 2.4 ± 0.3 moles of amino nitrogen were found to have been removed by deamination. By the FDNB procedure, less than 0.05 mole of α,ϵ-bis DNP-lysine was recovered. Native RNase, carried through the FDNB procedure, was assumed to yield 1 mole of α,ϵ-bis DNP-lysine per mole and was used to correct for hydrolysis and chromatography losses. No α-hydroxy ϵ-DNP-lysine was found. Thus, the 2 amino groups removed by deamination appeared to be from the N-terminal lysine. About 0.5 mole of additional amino groups was absent. The peptides from a trypsin digest

* Numbers in parentheses refer to location of the amino acid in the chain.

of performate-oxidized deaminated RNase were compared with those from a control that had not been deaminated. Peaks corresponding to all of the major peptides found by Hirs, Moore, and Stein[21] were found in both preparations, except that peptide No. 10, which should contain the N-terminal lysine, was not recovered from the deaminated preparation. Since trypsin should not produce a given peptide if the potential C-terminal residue is ε-OH-

TABLE 2

RNASE AND DEAMINATED RNASE PRECIPITIN ANALYSIS*

	Tube No.								
	1	2	3	4	5	6	7	8	9
Ra-a-RNase, B343-1, ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Deaminated RNase, Pool I μg.N/ml.	2.0	4.0	6.0	8.0					
RNase, μg.N/ml.					2.0	4.0	6.0	8.0	
Total μg.N precipitated	35.0	40.0	32.0	15.0	39.0	72.0	89.0	65.0	13.0

* The reactions were done in duplicate, reacted 0° C. for 72 hours, washed 3 times with saline, and analyzed for nitrogen.

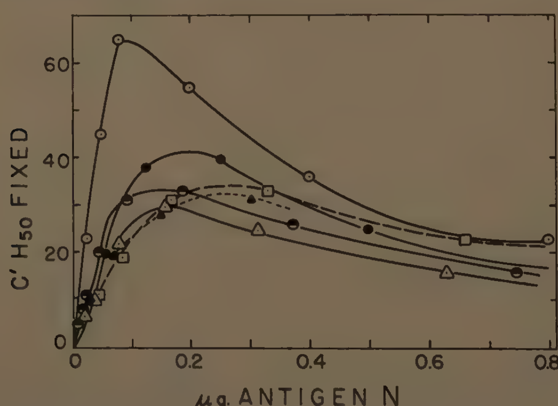

FIGURE 2. Complement fixation with various RNase derivatives. Native RNase is represented by an *open circle;* guanidinated RNase, a *closed circle;* acetylated guanidinated RNase, a *square;* deaminated guanidinated RNase, a *half-closed circle;* PTC guanidinated RNase, an *open triangle;* and deaminated RNase carried through the Edman procedure, a *closed triangle.*

lysine, the recovery of all peptides, except No. 10, suggested that this deaminated RNase consisted of an α-ε-diOH-lysine in the N-terminal region with a small amount of random deamination of the other amino groups. Although nitration is a frequent side reaction of deamination, none was detected spectrophotometrically in these samples. Deaminated RNase reacted only partially with antibody to RNase, as judged by C′ fixation or precipitin analyses (TABLE 2).

Guanidinated RNase (G-RNase) was also used to study the role of the α-amino group. As judged by recovery of 1 mole of DNP homoarginine per

mole of RNase, *O*-methylisourea did not react with this group. The enzyme was guanidinated, using the conditions for human serum albumin.[22] Aliquots of the reaction mixture were dialyzed, hydrolyzed, and passed through an automatic amino acid-analyzing device of the type described by Spackman *et al.*[23] The material used for the studies described below contained less than 0.1 mole of lysine per mole of RNase. Klee and Richards[24] examined preparations of this type, and concluded that lysine is the only amino acid attacked. G-RNase is not chromatographically separable from native RNase, and reacts almost as well with antibody (FIGURE 2). G-RNase was treated with nitrous acid, acetic anhydride, or phenylisothiocyanate. All 3 derivatives had a simi-

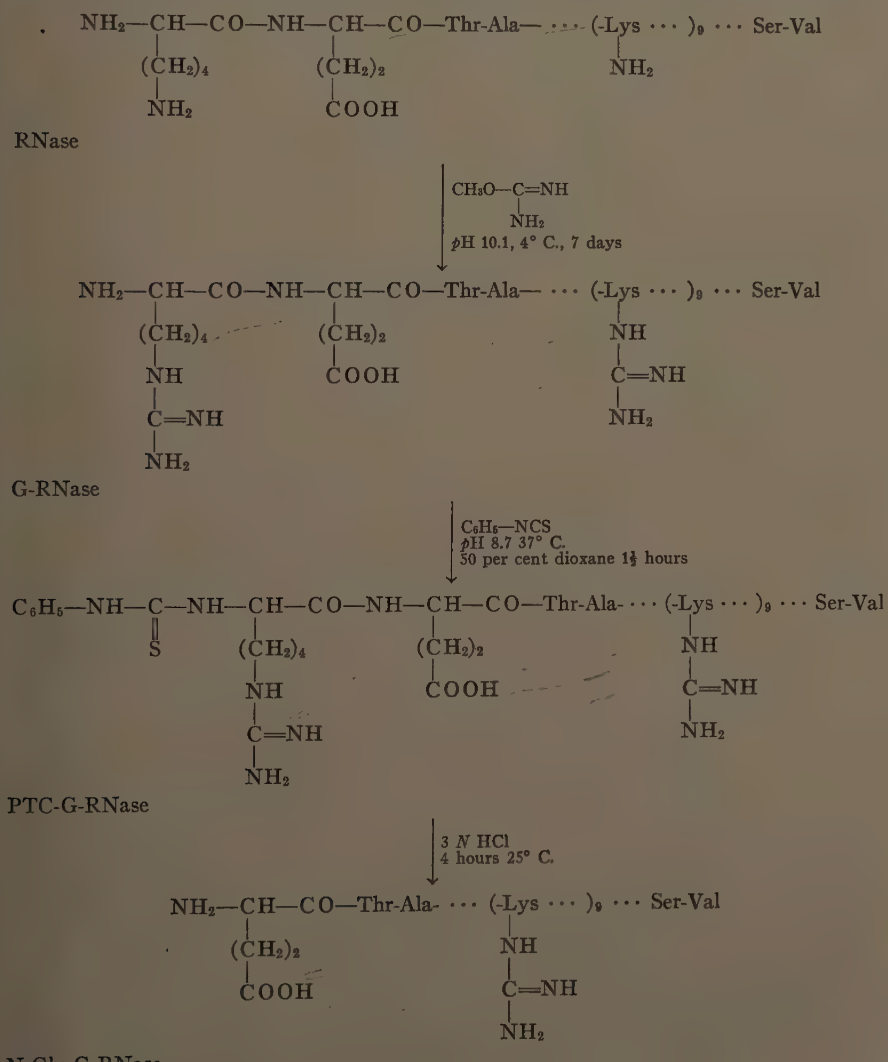

DIAGRAM 1.

lar loss of reactivity by C′ fixation (FIGURE 2). The amounts of these substances available were too small for further purification, and inert materials probably account for the minor shift in peak fixation. The variety of reagents used makes it unlikely that side reactions accounted for the observed loss of immune reactivity and indicates that the α-amino group of the N-terminal lysine is essential for immune reactivity.

Geschwind and Li[25] have suggested the Edman degradation of guanidinated proteins. The phenylthiocarbamate of G-RNase (PTC-G-RNase) was hydrolyzed to remove the entire lysine residue and leave an RNase derivative with an N-terminal glutamic acid by the Edman procedure (see DIAGRAM 1).

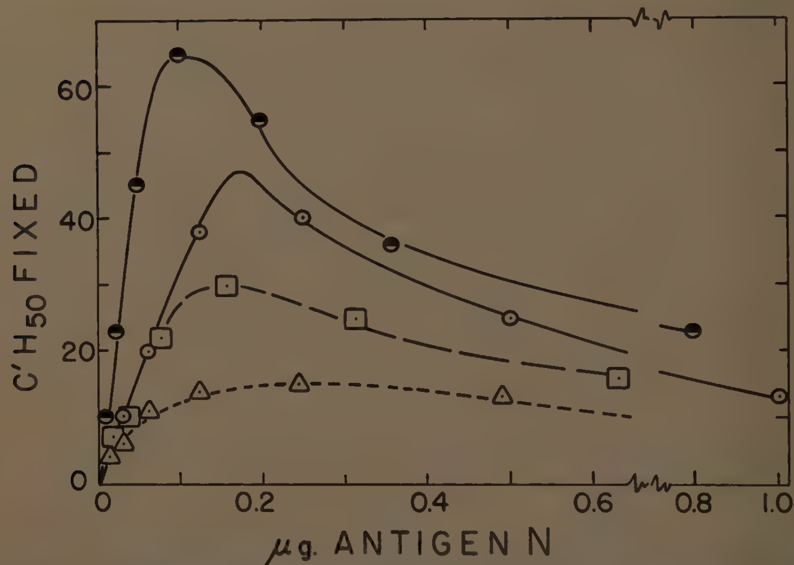

FIGURE 3. Complement fixation with *N*-Glu-G-RNase (*triangle*) and its precursors native RNase (*half-closed circle*), guanidinated RNase (*open circle*), and PTC guanidinated RNase (*square*).

The N-terminal glutamic guanidinated RNase (N-Glu-G-RNase) was chromatographed on IRC-50 at *p*H 6.16 in 0.2 M phosphate buffer. A single peak was obtained and tested for immune activity. N-Glu-G-RNase is much less reactive than PTC-G-RNase which, in turn, is less reactive than G-RNase by C′ fixation (FIGURE 3).

The following controls were run. Deaminated G-RNase was treated with PTC and hydrolyzed in the same manner as G-RNase. Since this substance does not have any amino groups, it should be unaltered unless side reactions are responsible for the decreased reactivity of *N*-Glu-G-RNase. Deaminated G-RNase treated in this manner retained its immunological reactivity, fixing 33 $C'H_{50}$ initially and 32 $C'H_{50}$ after treatment (FIGURE 2). Slightly more antigen was required to reach peak fixation after treatment, which probably reflects a small amount of destruction during the process. For another control, phenylisothiocyanate was omitted, but all other steps were carried out. G-

RNase treated in this manner had unaltered reactivity. Thus, the decreased reactivity of N-Glu-G-RNase does not appear to be due to side reactions. Chemical studies of the derivative reinforce this view. By the FDNB procedure, 0.9 ± 0.1 mole of N-terminal glutamic acid was recovered per mole of N-Glu-G-RNase. No bis DNP-lysine, ϵ-DNP-lysine, DNP-homoarginine, or other DNP amino acids were found, indicating that the reactions with *O*-methylisourea and phenylisothiocyanate were complete and that the hydrolysis was specific. The material can be carried through another cycle of Edman degradation, yielding N-terminal threonine guanidinated RNase (N-Thr-G-RNase), which is even less reactive toward antibody. Thus far, N-Thr-G-RNase has been characterized only qualitatively regarding its N-terminal amino acid.

Physicochemical and Enzymic Studies

In several of the above preparations, alteration of a specific group changed the reactivity of RNase to antibody. This group might react with the antibody or might link portions of the RNase molecule so that other portions that react with antibody are in proper orientation. These possibilities are portrayed schematically in FIGURE 4. If the N-terminal region of the original molecule (FIGURE 4*a*) did not unfold upon removal of the N-terminal lysine residue, N-Glu-G-RNase would have nearly the same shape as RNase. If unfolding occurred, N-Glu-G-RNase might look like the structure shown in FIGURE 4*b*. It is not now possible to choose between these alternatives. The observed stepwise loss of reactivity suggests union of antibody with the altered groups.

Marked unfolding might be detected by physical-chemical studies, although it is probable that minor unfolding could escape notice. Sedimentation constants of several derivatives were measured (TABLE 3) and showed little change from that of native RNase. Although no evidence of intramolecular reorientation was found, it is possible that more sensitive methods, such as optical rotation, would show such changes.

Studies of the enzymic activity of RNase derivatives may provide information about some of the structural features required. A certain orientation of the peptide chains is probably necessary. Retention of activity in a derivative implies that at least some chains are not disoriented.

G-RNase and its derivatives are almost devoid of enzymic activity. To study the role of the N-terminal lysine, a partially guanidinated (5.4 mole of homoarginine per mole) RNase was prepared. By Anfinsen's method,[9] it retained 46 per cent of its original enzymic activity on a nitrogen basis. The N-Glu-derivative was prepared in the same manner as N-Glu-G-RNase. The chromatographic behavior (FIGURE 5) and reaction with antibody were similar to those of N-Glu-G-RNase. Precipitin studies also showed a marked decrease in the reaction with antibody (FIGURE 6). Glutamic acid (0.9 mole) was the only N-terminal amino acid present. Analyses for homoarginine gave the same value as the starting material, but the lysine content was about 1 mole lower, suggesting that 1 ϵ-amino group might have reacted with phenylisothiocyanate. By enzymic assay, the material was 41 per cent as active as native RNase. Thus, only a small amount of enzymic activity, if any, was lost by removing the N-terminal amino acid.

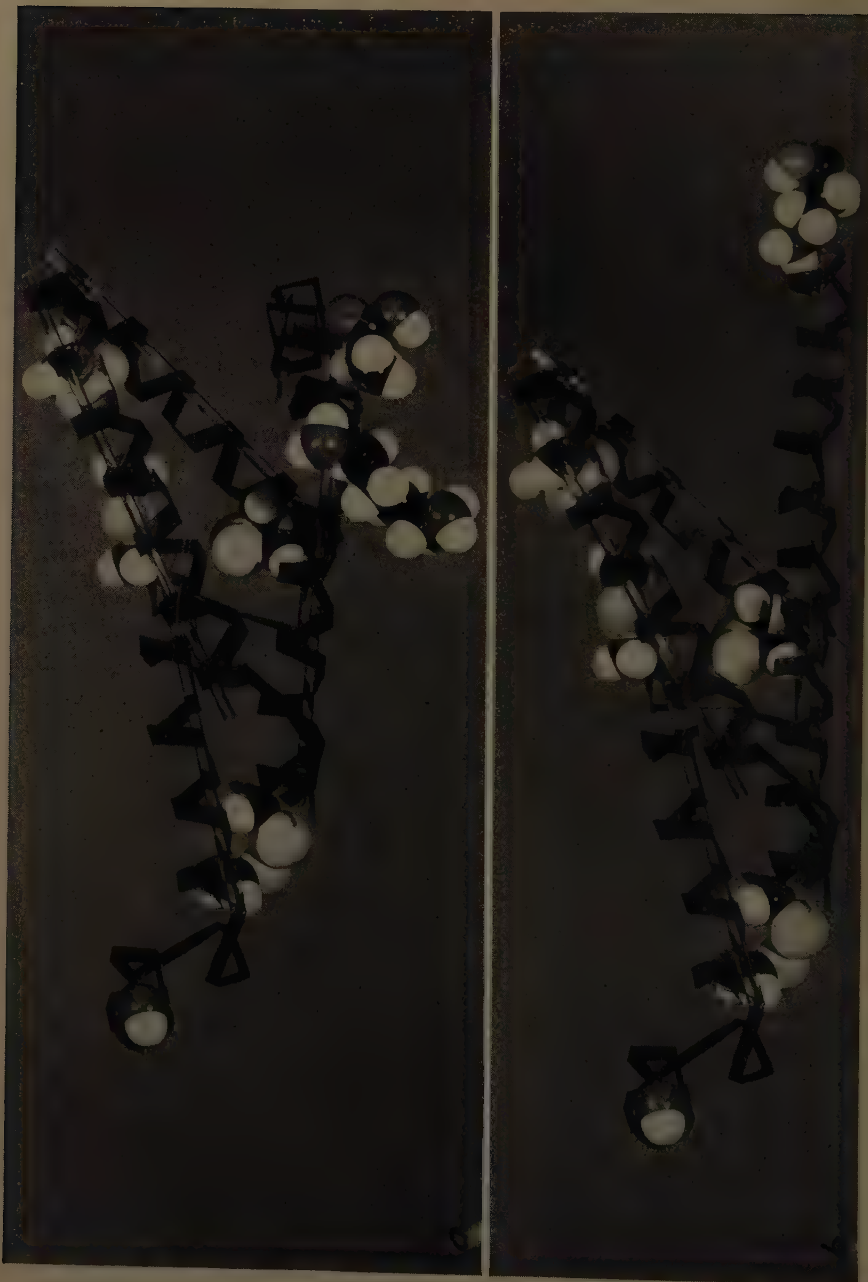

FIGURE 4(*a*). Schematic diagram of a possible structure for native RNase and (*b*) an unfolded N-Glu-G-RNase.

Table 3

Physical, Enzymic and Immunological Properties of Some RNase Derivatives

Derivative	$S_{20,w} \times 10^{13}$	Enzyme activity* (per cent)	Immune reactivity† (per cent)
Native	1.89	100	100
Deaminated	1.87	130	40
Guanidinated	—	<1	86
Deaminated guanidinated	—	4	51
Acetylated guanidinated	—	7	50
PTC guanidinated	—	—	45
N-Glu-guanidinated	1.79	—	24
Partially guanidinated	—	46	95
Partially guanidinated N-Glu	1.89	41	29
Acetylated	1.74	3	<1
Alkaline denatured (not chromatographed)	—	—	63
Reduced and treated with iodoacetic acid	—	5	52

* Activity compared to native RNase by the procedure of Anfinsen. Dashes indicate no assays done.

† Ratio of maximum $C'H_{50}$ fixed times 100 to maximum $C'H_{50}$ fixed by native RNase.

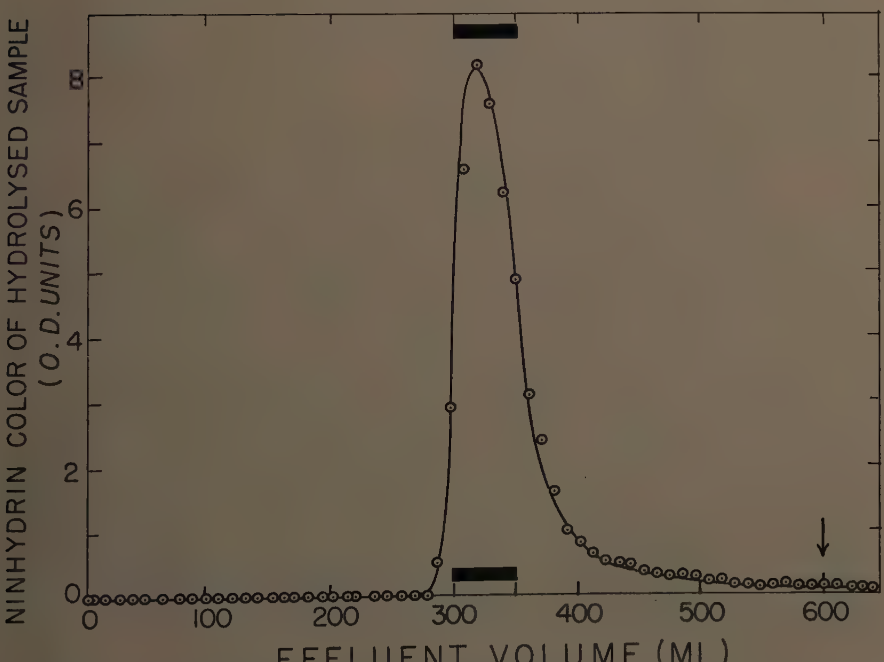

Figure 5. Chromatography of partially guanidinated N-Glu-RNase on IRC-50 at *p*H 6.26 with 0.2 M phosphate buffer on a 48- × 3.6-cm. column.

Deaminated RNase showed some anomalies on enzymic assay. Deaminated RNase always had 20 to 40 per cent more activity, by the Anfinsen[9] and Kunitz[26] assays, than native RNase. Activity by Richards' method,[27] using 2′,3′ cyclic cytidine phosphate as substrate at *p*H 5, was almost identical for RNase and deaminated RNase at ionic strengths of 0.042, 0.083, 0.17, and 0.42 and substrate concentrations of 3.42, 6.85, 10.1, 13.7, and 27.3 mmoles per liter. Perhaps deaminated RNase exhibits some dissociation of depolymerase and cyclic cytidine phosphatase (CCPase) activities.

The retention of immune reactivity and loss of enzymic activity in G-RNase and the decrease of immune reactivity with little additional loss of enzymic

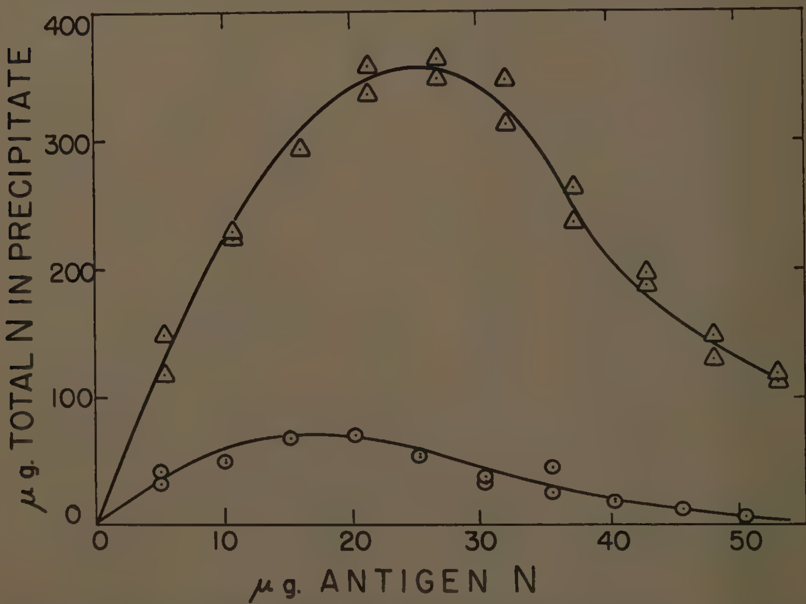

FIGURE 6. Amount of specific precipitate formed from various amounts of RNase and partially guanidinated N-Glu-RNase reacting with 1 ml. of rabbit antibody.

activity during formation of partially guanidinated *N*-Glu-G-RNase suggested that the antigenic and enzymic sites were not identical.

Specific precipitates of RNase-a-RNase had little enzymic activity toward ribonucleic acid, unless the antibody had been denatured by urea treatment. Assays done acid to *p*H 5 also showed partial recovery of enzyme activity, probaby due to dissociation of the specific precipitate. CCPase activity was only slightly diminished by antibody. Steric hindrance of the large RNA molecule by antibody may account for the inhibition, although interaction of a portion of the depolymerase site not common to the CCPase site might also be involved.

Human RNase

A study of the amino acid sequence of any ribonuclease that cross-reacts completely with the antibody to bovine RNase should help elucidate the com-

bining sites of the antigen. Human male urine was fractionated by means of ethanol and ammonium sulfate precipitations,[28] followed by chromatography on carboxymethylcellulose and IRC-50 resins. The RNase isolated by these means had 1 mole of N-terminal lysine and no other end groups by the FDNB procedure, and a sedimentation constant identical with that of bovine RNase. Free electrophoresis at *p*H 8.6 yielded a single boundary that moved toward the negative pole. The human RNase reacted completely with rabbit anti-bovine RNase by quantitative complement fixation, although a tenfold excess

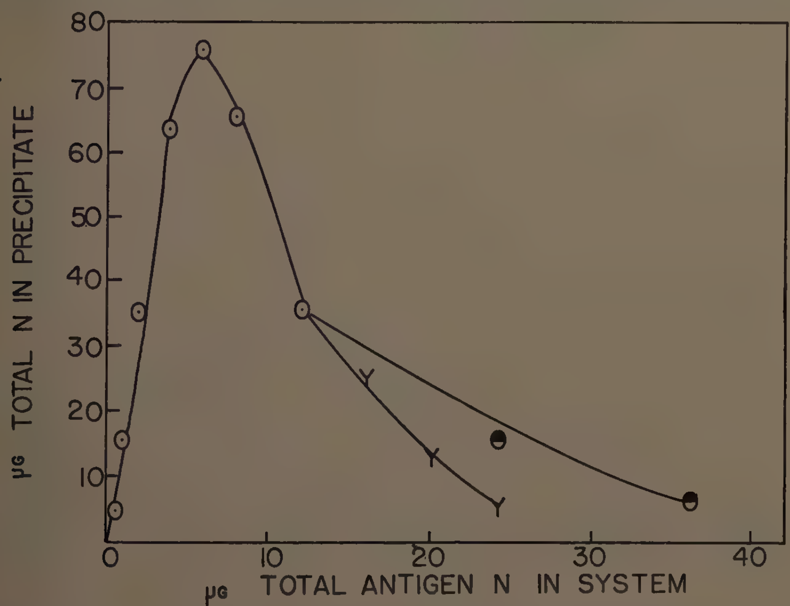

FIGURE 7. Amount of specific precipitate formed from increasing amounts of RNase (*circle*) and 1 ml. of rabbit antibody. Precipitates with 12 µg. of RNase N were reacted with an additional 4, 8, or 12 µg. of RNase N (Y) or 12 or 24 µg. of acetylated RNase N (*half-closed circle*), and the amount of N in the precipitates determined.

of enzyme activity over the bovine was required. Further studies on the amino acid sequence are planned.

Classification of Structural Features into Specific Sites

The Goldberg equation[29] was used to calculate the valence of the antigen from the mole ratio of RNase to a-RNase in the specific precipitate and in the entire system. RNase had a valence of 2 or 3 with these antisera. Partially acetylated RNase (Acet RNase) does not precipitate with some sera, but is an effective inhibitor of precipitation (FIGURE 7). The dotted lines in the figure suggest that 10 mg. of Acet RNase will dissolve as much precipitate as 5 mg. of native RNase in antigen excess; Acet RNase may be a univalent antigen. Agglutination experiments with erythrocytes coated with RNase or Acet RNase are consistent with this view. A serum that gave no precipitate with Acet RNase agglutinated tanned erythrocytes coated with Acet RNase, as well as

it agglutinated RNase-coated cells. If one assumes an antigen valence of 2, the essential structures may be classified. A derivative that had poor reactivity was acetylated and tested as an inhibitor. It is placed in Class 1 if it is still a good inhibitor, and it is assumed that the initial alteration affected the same site as would acetylation. Derivatives in Class 2 are poor inhibitors after acetylation, suggesting that both sites have been altered; that is, the initial alteration is on the site not affected by acetylation. This classification is tentative and may require modification. One or both sites may require subdivision if the antigen valence is greater than 2. Some structures may be common to both sites, or the antibody may be so heterogeneous that the sites become ill defined. TABLE 4, which also serves as a summary, classifies the

TABLE 4
TENTATIVE CLASSIFICATION OF STRUCTURES STUDIED IN THE REACTION OF RNASE WITH ITS ANTIBODY

Nonessential structures
C-terminal valine (124)
OH or NH_2 of the carboxyl group differing in RNase *A* and *B*
Any irreversibly altered nontyrosyl hydrogen bonds
Essential structures unclassified as to site
One to three tyrosyl-hydrogen bonds or the structures they stabilize
One or more of the peptide bonds cleaved by pepsin
One or more of the peptide bonds cleaved by chymotrypsin
Histidine (116)
N-terminal lysine (1) and adjacent glutamic acid (2) residues
Essential structures of Site 1
One or more disulfide bonds
α-amino group of N-terminal lysine (1)
One or more ϵ-amino groups or residues of similar charge
Essential structures of Site 2
One or more disulfide bonds
Peptide bond 120, a portion of the molecule between this and bond 123, or both

structures studied thus far. The data on histidine (116) are from the work of Weil and Seibles,[30] who showed that loss of enzyme activity paralleled the destruction of 1 histidine; from the work of Stein and Barnard,[31] who showed that histidine (116) was attacked by bromacetic acid, destroying enzyme activity; and from the observation that photo-oxidation causes concomitant loss of enzymic and immune reactivity.

Acknowledgment

Much of the work concerning hydrogen and disulfide bonds,[32] deamination,[33] and acetylation was done in collaboration with Lawrence Levine and Helen Van Vunakis, both now at Brandeis University, Waltham, Mass. Detailed descriptions of these portions are in preparation. Their experiments and the concomitant discussions formed the basis for the more recent studies. We also thank C. B. Anfinsen for the pepsin-treated RNase derivative described in *Peptide Bonds*.

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Discussion

A. A. Hakim (*Miami Heart Institute, Miami Beach, Fla.*): Enzymic heterogeneity of crystalline ribonuclease has been described by Hakim.[1-4] In these studies crystalline ribonuclease samples obtained from different sources, as well as a sample prepared from calf pancreas in the laboratory, were separated by column chromatography into ribonuclease *A* and ribonuclease *B*.

Ribonuclease *B* of each of the four crystalline ribonucleases liberates guanylic acid from ribonucleic acid, and it catalyzes synthesis of dinucleoside phosphates from cyclic cytidylic, uridylic, and guanylic acids, but not from cyclic adenylic acid. Ribonuclease *B* fraction, similar to the ribonuclease *A* fraction and to the crystalline ribonuclease, hydrolyzes ribonucleic acid, liberating pyrimidine mononucleotides. It also hydrolyzes cyclic guanylic acid to guanosine 3′-phosphate, whereas ribonuclease does not.

Further attempts in the study of ribonuclease heterogeneity and specific activity[3, 5] were made on two-dimensional paper electrophoresis. Crystalline ribonuclease was resolved into four enzymically active fractions: I, II, III, and

IV. TABLE 1 shows the ribonuclease activity of the four fractions in each of the five crystalline enzymes in two-dimensional paper electrophoresis. Each of the four fractions failed to act on diphenyl phosphate.

TABLE 1
RIBONUCLEASE ACTIVITY OF THE DIFFERENT ACTIVE FRACTIONS RESOLVED BY TWO-DIMENSIONAL PAPER ELECTROPHORESIS

	Original activity (per cent)	Fractions resolved by paper electrophoresis				Recovery (per cent)
		I	II	III	IV	
Armour RNase	100	48	41	6.5	2.5	98
Worthington RNase	100	49	43	3.0	2.0	97
Nutritional Biochemicals RNase	100	50	44	1.0	0	95
Laboratory RNase	100	49	41	6.0	1.0	97

TABLE 2
ACTION OF CRYSTALLINE RIBONUCLEASE FRACTIONS ON SYNTHETIC SUBSTRATES

Fraction	Cytidine 2′,3′-phosphate		Uridine 2′,3′-phosphate		Adenosine 2′,3′-phosphate		Guanosine 2′,3′-phosphate	
	Extent of hydrolysis (percentage)	Reduction products	Extent of hydrolysis (percentage)	Reaction products	Extent of hydrolysis (percentage)	Reaction products	Extent of hydrolysis (percentage)	Reaction products
I	60	CMP-3′*	80	UMP-3′*	0	—	0	—
II	78	CMP-3′	65	UMP-3′	0	—	0	—
III	45	CMP-3′ (-2′)	40	UMP-3′ (-2′)	60	AMP-3′*	15	GMP-3′
IV	10	CMP-3′ (-2′)	15	UMP-3′ (-2′)	20	AMP-3′	55	GMP-3′

* CMP-3′, UMP-3′, AMP-3′, and GMP-3′ are the 3′-derivatives of cytidylic, uridylic, adenylic, and guanylic acid, respectively.

TABLE 3
ACTION OF CRYSTALLINE RIBONUCLEASE FRACTIONS ON YEAST RIBONCULEIC ACID

Fractions	Mononucleotides liberated (moles per 100 moles)			
	Uridylic acid	Cytidylic acid	Adenylic acid	Guanylic acid
I	17.6	17.4	0.5	0.5
II	17.0	18.5	2.5	0.5
III	15.6	14.5	8.5	2.5
IV	11.5	10.5	4.5	10.5

Differences in the activity of the fractions I, II, III, and IV on the cyclic anhydrides are also shown in TABLE 2.

Ribonuclease Fractions I and II demonstrated greater enzymic activity on uridine 2′,3′-phosphate and cytidine 2′,3′-phosphate than either Fraction III or Fraction IV. Consequently, Fractions III and IV showed a greater enzymic

activity on adenosine 2′,3′-phosphate and guanosine 2′,3′-phosphate than either Fraction I or II.

The enzymic action of the four components of ribonuclease on ribonucleic acid revealed quantitative differences in mononucleotides liberated (TABLE 3). Ribonuclease Fraction IV liberated more guanylic acid (10.5 moles/100 moles) than Fraction III (2.5 moles/100 moles), Fraction II (0.5 mole/100 moles), or Fraction I (0.5 mole/100 moles). Fraction III liberated more adenylic acid (8.5 moles/100 moles) than Fraction IV (4.5 moles/100 moles), Fraction II (2.5 moles/100 moles), or Fraction I (0.5 moles/100 moles). Fraction II liberated more cytidylic acid (18.5 moles/100 moles) than Fraction I (17.4 moles/100 moles), Fraction III (14.5 moles/100 moles), or Fraction IV (10.5 moles/100 moles).

All attempts to resolve RNase *A* or RNase *B* have failed, as crystalline ribonuclease was resolved into enzymically active fractions on two-dimensional paper electrophoresis. RNase *A* and RNase *B*, after passage through ion exchange resin, were not resolvable. It is therefore evident that the structure of RNase *A* or RNase *B* was altered in an unknown manner. Does this structural alteration influence the enzymic specificity of the two protein fractions? Is the difference in the specific enzymic activity of the four enzymically active fractions resolved by two-dimensional paper electrophoresis due to the splitting off of certain potentially active interlinkages, or is it due to the number of active groups in each of the fractions? Although it is still too early to draw any mature conclusions, we are faced with the possibility that the four enzymically active fractions obtained by two-dimensional paper electrophoresis (if they do not represent four different ribonucleases) are active fractions with different specific activity obtained from one crystalline enzyme.

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THE RELATION OF STRUCTURE TO ENZYMATIC ACTIVITY IN RIBONUCLEASE*

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Before proceeding with our experiments relating structure to function in the enzyme bovine pancreatic ribonuclease (RNase) and before attempting to isolate an active fragment of RNase containing an active center (if it exists), we felt it necessary to improve the methods of assaying for RNase.

The first part of this presentation, therefore, will deal with the assay procedures employed and with the effects of salts and urea on these procedures and on the substrate, yeast sodium ribonucleate (YRNA). The second part will attempt to provide answers to the following questions relating the structure of the ribonuclease molecule to its enzymatic activity: (1) Are the 4 disulfide bonds necessary for enzyme activity? (2) Is the tightly coiled form of the molecule essential for its activity? (3) Is the whole, intact protein essential for its catalytic activity?

Part 1

Ribonuclease assays. Commercial ribonucleic acid (RNA) is probably suitable for routine assays but, from a chemical and esthetic point of view, it is unsatisfactory to work with the brownish, murky solutions that result when commercial RNA preparations are dissolved in water. In order to avoid these and, in an attempt to improve the assay procedure, we isolated our RNA from yeast according to the method of Crestfield *et al.*[1] This YRNA is a clean, white, fluffy powder and gives clear, colorless, viscous solutions when dissolved in water. It is more rapidly hydrolyzed to acid-soluble oligonucleotides by pancreatic RNase and gives smaller blank values than commercial preparations.

A standard assay curve for the spectrophotometric assay of RNase, using this YRNA, is presented in FIGURE 1. The assay is essentially a modification of previously published procedures by MacFayden,[2] Kunitz,[3] and Anfinsen *et al.*,[4] and is based on the liberation of acid-soluble oligonucleotides from YRNA. The test tubes contain 1.0 ml. of 0.1 M acetate buffer, *p*H 5.0 (0.065μ) + 0.01 ml. of RNase solution. To this is added 1 ml. of 1 per cent YRNA solution in acetate buffer. This mixture is incubated for 4 min. at 37° C., at which time 1 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid is added. The mixture is cooled in an ice bath and then centrifuged at 3° C. Aliquots (0.1 ml.) of the supernatant are promptly diluted with 3 ml. of water and the absorbancy of the acid-soluble oligonucleotides is measured in a Beckman DU Spectrophotometer in square, 1-cm. silica cuvettes at 260 mμ. Each

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point in FIGURE 1 represents the average of 4 consecutive experiments chosen at random; arrows indicate the extent of variation in the method. There is a straight-line relationship between activity and micrograms of RNase to 8 μg. of the enzyme. In addition to the slight variation, a slight disproportionality is observed at the origin—that is, the straight line does not go through the origin. We do not know whether this break is real; it may be due to absorption of small amounts of the enzyme protein on the surface of the glass tubes. It may be an indication that the YRNA substrate undergoes some reaction

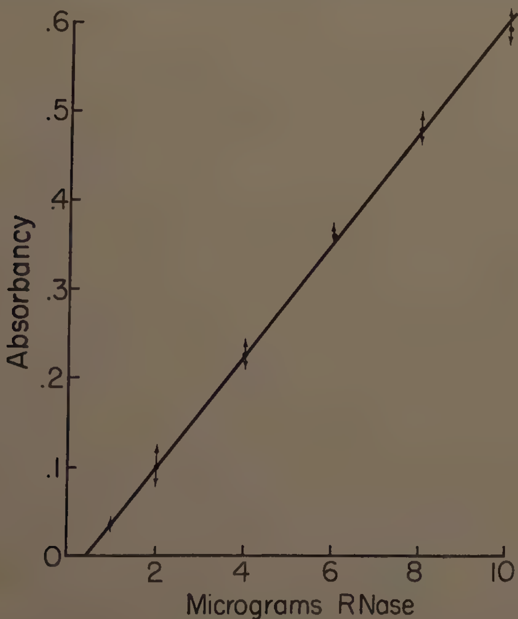

FIGURE 1. Standard curve for spectrophotometric assay of RNase activity at *p*H 5.0. Conditions: 2 ml. of 0.1 M acetate buffer, *p*H 5.0 (μ = 0.065), 0.01 ml. of RNase solution, and 1 ml. of 1 per cent RNA in buffer, incubated 4 min. at 37° C. Then 1 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid was added and centrifuged at 3° C., and 0.1-ml. aliquots of the supernatant substance was diluted with 3 ml. of water and read at 260 mμ.

such as a denaturation before being digested by RNase, or it may be due to the fact that the initial products of RNase action are still of large molecular weight and are not soluble in acid. With care and clean glassware, the method is sensitive and reliable. However, its sensitivity can be increased tenfold by carrying out the assay at *p*H 7 (FIGURE 2). Under these conditions, 2.0 ml. of 0.1 M phosphate buffer (*p*H 7.3, 0.254 μ) are incubated with 1 ml. of 2 per cent YRNA solution (in water) for 4 min. at 30° C. The reaction is then stopped with uranyl acetate–perchloric acid reagent, and the assay continued as described above for *p*H 5.0. Each point is an average of 5 to 10 experiments; it may be seen that the product obtained from yeast according to the method of Crestfield *et al.*[1] is a more effective substrate than the commercial preparation.

RNase activity was also measured from the rate of acid formation at constant pH, using a Radiometer TTT-1 pHstat, with either YRNA or cytidine 2′,3′-cyclic phosphate (Cp!) as substrate (FIGURE 3).

The effects of salts and urea on the assay procedure. The ionic requirement for RNase activity was found to vary with pH (FIGURE 4), the optimum being 0.2 μ at pH 5.2 and 0.06 to 0.1 μ at pH 7.3. However, optimum ionic requirements for maximum activity did not produce linear assays; the conditions described

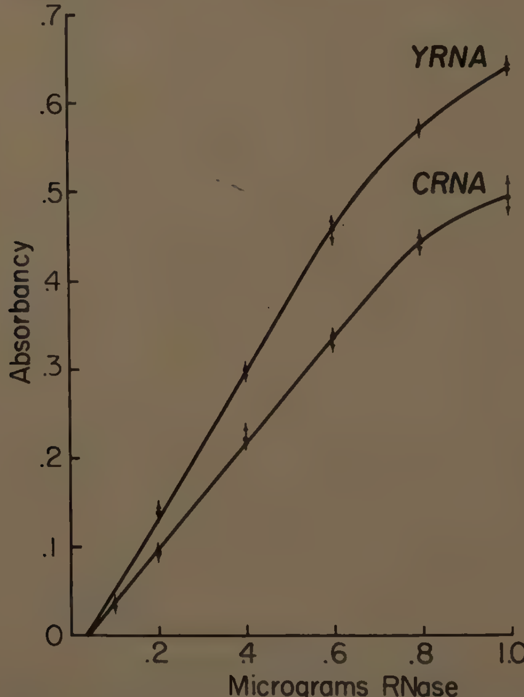

FIGURE 2. Standard curve for spectrophotometric assay of RNase activity at pH 7.3. Conditions: 2 ml. of 0.1 M phosphate buffer, pH 7.3 (μ = 0.254), 0.01 ml. of RNase, and 1 ml. of 2 per cent RNA (in water) incubated 4 min. at 30° C. Other conditions were the same as is FIGURE 1.

for FIGURES 1 and 2 did. The optima for pH and ionic strength were interdependent, the optimum shifting toward a lower pH with increasing salt concentrations (FIGURE 5).

The sodium ribonucleate extracted from yeast by the method of Crestfield *et al.*[1] is a surprisingly stable substrate. One per cent solutions can withstand heating for 1 hour at 75° C., shaking for 24 hours, 1-hour aeration, or storage for 2 days at room temperature or to 7 days in the refrigerator with little or no effect on the blank values or on its ability to act as a substrate for RNase. Ultraviolet irradiation for 1 hour leads to an approximate one-third loss in activity. However, treatment of YRNA at pH 12.3 for 30 min., followed by precipitation with acid and neutralization to pH 6.2, resulted in a surprising

increased ability to be digested by RNase; treatment with urea produced similar results. FIGURE 6 shows the effect of different (final) concentrations of urea on RNase activity. In each case, YRNA was dissolved in varying concentrations of urea. One milliliter of each of these solutions was then added to 1.5 ml. of phosphate buffer, containing 0.2 to 1.0 μg. of the enzyme, and the spectro-

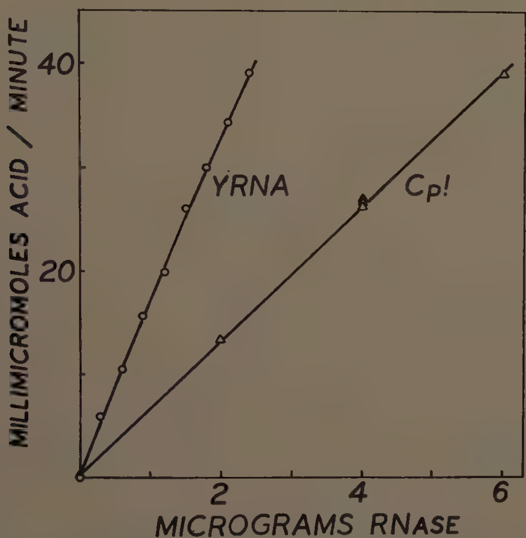

FIGURE 3. Standard curve for acidimetric assay of RNase activity. Vertical axis is the rate of addition of 0.5 N NaOH between 2 and 4 min. after addition of RNase to 5 mg. of RNA (*circles*) or 19.2 μmoles of Cp! (*triangles*) in 1.5 ml. of 0.1 M KCl at 25° C. and pH 7.0 under N_2 atmosphere.

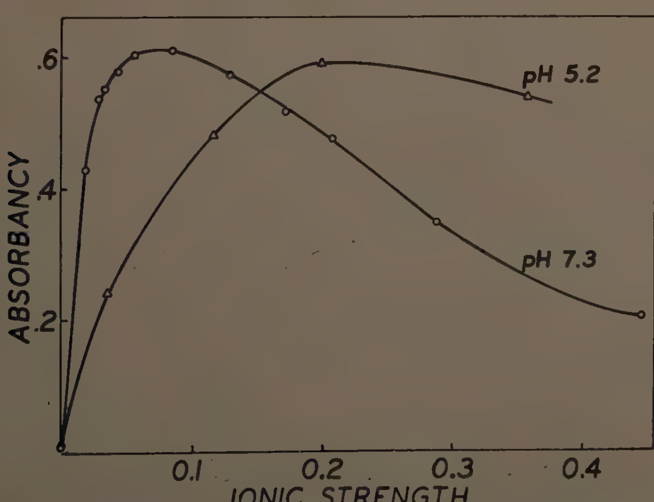

FIGURE 4. The influence of variations in ionic strength upon RNase activity as measured spectrophotometrically at pH 5.2 and 7.3 .

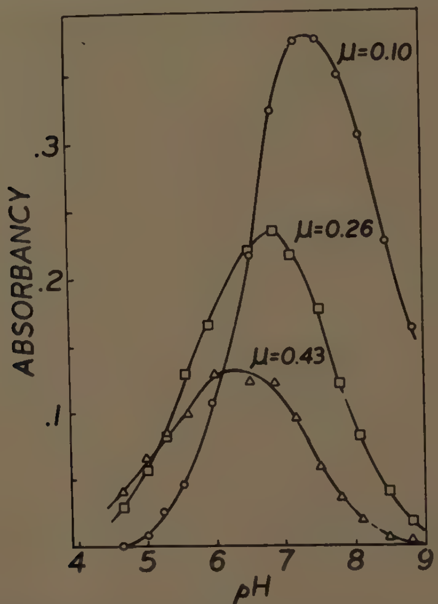

FIGURE 5. The influence of different ionic strengths upon the pH optimum of RNase as measured spectrophotometrically.

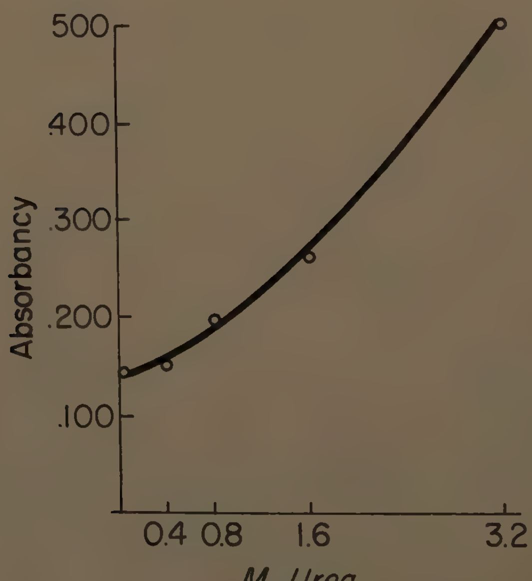

FIGURE 6. The effect of various concentrations of urea upon the spectrophotometric measurement of RNase activity. Conditions: 10 mg. of RNA and 0.6 μg. of RNase in 2.5 ml. of 0.1 M phosphate, pH 7.0, were incubated 2 min. at 30° C. with added urea.

photometric assay was carried out for 2 min. at 30° C. Appropriate blanks were carried out for all of these solutions without the enzyme. It is quite clear from these experiments that the absorbancy increases with increasing concentrations of urea. The effect of urea on the spectrophotometric assay of RNase at *p*H 7.0 is presented in FIGURE 7. When 1 ml. of a 1 per cent YRNA solution, dissolved in 8 M urea, is added to 1.5 ml. of the enzyme in phosphate buffer, a large increase in acid-soluble oligonucleotides is obtained that is largely pro-

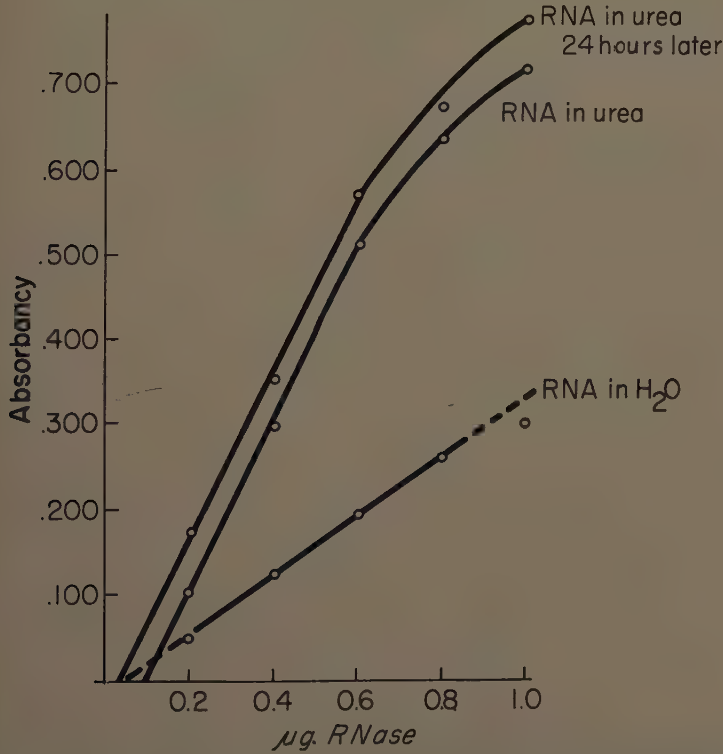

FIGURE 7. The influence of urea upon the spectrophotometric assay of RNase activity Conditions: 10 mg. of RNA in 2.5 ml. of 0.1 M phosphate, *p*H 7.0, were incubated with RNase at 30° C. for 2 min. with or without added urea (final concentration 3.2 M).

portional to the amount of enzyme present. A slightly greater increase was obtained when the YRNA-urea solution was tested 24 hours later (urea increases the blank values by about 0.07 optical density units over those obtained with water). The effect with urea is *completely reversible* on dialysis. Urea exerts its greatest effect at neutral or slightly acid *p*H (FIGURE 8), but does not appear to change the optimum *p*H for ribonuclease activity under the conditions of these experiments.

There appear to be at least two possible explanations for the urea effect on this system. The first is that urea disrupts some of the hydrogen-bonded structure of sodium ribonucleate, denatures it and, therefore, makes it more available

to subsequent digestion by the enzyme. The second is that urea tends to solubilize the products of the enzyme action. We believe that both these processes are taking place, and evidence for this is presented in TABLE 1. With no pretreatment of YRNA, activity under these conditions is low. Whether one pretreats the substrate or the enzyme with urea, or whether one adds the

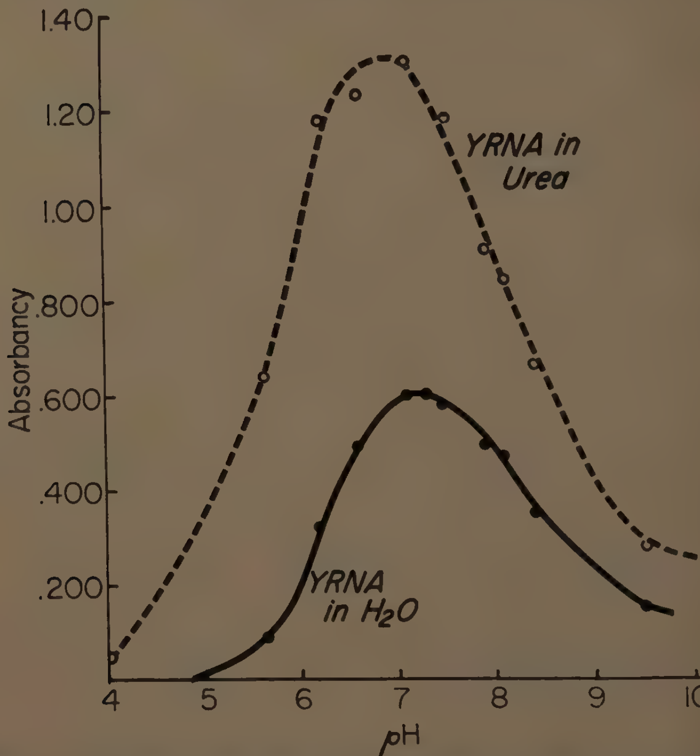

FIGURE 8. The effect of urea upon the *p*H optimum of RNase activity as measured spectrophotometrically. Conditions: 10 mg. of RNA and 2 μg. of RNase in 3 ml. of veronal-acetate buffer (μ = 0.123), in the absence or presence of 2.6 M urea, was incubated for 2 min. at 30° C. before addition of acid.

TABLE 1
ABSORBANCY VALUES WITH UREA TREATMENT IN RNASE ASSAY*

Urea treatment	Filtrate absorbancy (μg.)
None	0.06
Substrate preincubated with urea 3 hours	0.37
Enzyme preincubated with urea 15 min.	0.35
Urea and substrate added simultaneously to enzyme	0.36
Urea added 15 sec. before reaction stopped	0.15
Urea added after reaction stopped	0.11

* RNase (6 μg.) plus 10 mg. sodium ribonucleate (substrate) in 3 ml. 0.03 M acetate buffer (*p*H 5), plus 2.6 M urea. Time, 4 min.; temperature, 30° C.; treated with acid, centrifuged, and diluted as described in the text.

urea and substrate simultaneously to the enzyme does not seem to make much difference—the same increase in acid-soluble oligonucleotides is obtained. About one third of this absorbancy value can be accounted for by adding the urea immediately after or immediately before the reaction is stopped (by the addition of uranyl acetate–$HClO_4$ reagent). This shows the solubilizing effect of urea on the products of the reactions. If the reaction is stopped by the addition of an inhibitor (such as phenylisocyanate), and if urea is then added and allowed to incubate for 4 min., no further increase in the absorbancy values is

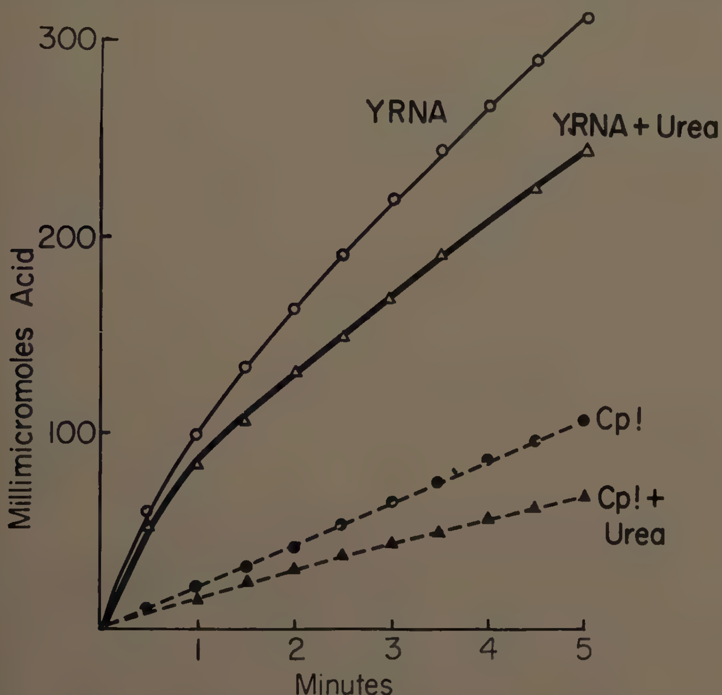

FIGURE 9. The inhibition of RNase activity by 3.2 M urea as measured titrimetrically. The ordinate is the 2- to 4-min. rate of alkali consumption to maintain a *p*H of 7.0 in a solution of 3 μg. of RNase and 6 mg. of RNA or 19.2 μmoles of Cp! in 1.5 ml. of 0.13 M KCl at 25° C.

obtained, indicating that the solubilizing effect of urea on the products of the reaction is immediate and not dependent on time.

This increased yield of acid-soluble oligonucleotides observed in the presence of urea is not a true stimulation of the enzyme, since urea inhibits RNase activity as measured by acid formation at constant *p*H in the *p*Hstat (FIGURE 9). The concentrations of sodium ribonucleate, urea, and enzyme in this case are the same as in the spectrophotometric assay. Percentagewise, urea inhibited the hydrolysis of 2′,3′-cyclic cytidylic acid to a greater extent than it inhibited the hydrolysis of YRNA.

The effects of salts and urea on YRNA. Since YRNA is itself an anionic polymer, it might be expected that the over-all charge and structure of the mole-

cule would influence its rate of digestion by RNase. In view of this, the effects of salts and of urea on some physical properties of RNA were investigated.

The initial relative viscosity of 1 per cent sodium ribonucleate solutions at 25° C. was high (provided stirring or mechanical agitation was avoided) and gradually decreased on standing (FIGURE 10). Urea (8 M) lowered the viscosity, as did 0.08 M KCl, but in a different manner. Urea plus KCl reduced the relative viscosity almost to unity. The more abrupt decrease in the relative viscosity with KCl, an electroviscous effect, appears to be similar to that

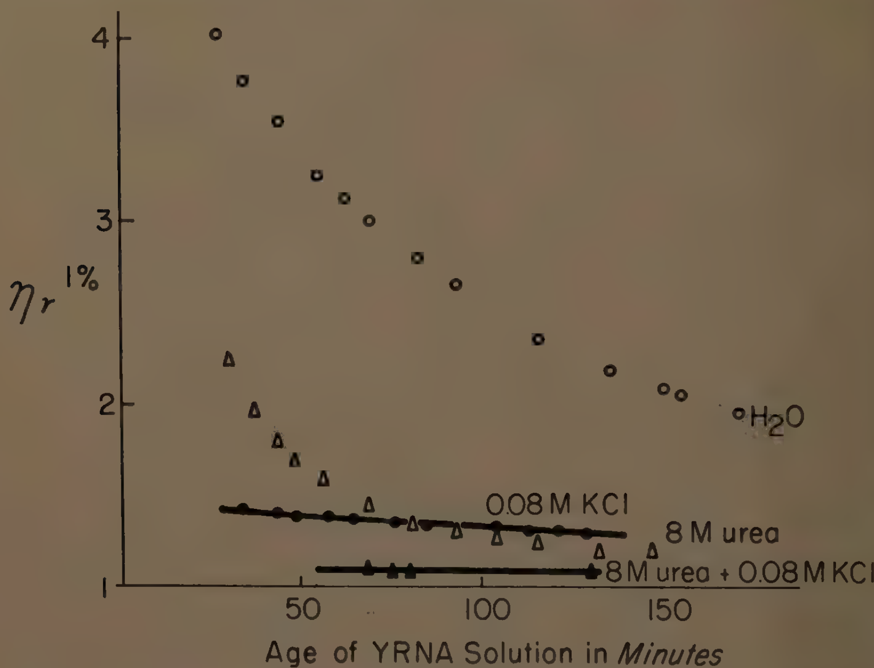

FIGURE 10. The effect of aging on the relative viscosity of 1 per cent RNA in water and dilute KCl, or in concentrated urea solutions, or both.

caused by addition of electrolytes to other highly ionized polymers, such as polyacrylic acid,[5] proteins,[6, 7] and desoxyribonucleic acid,[8-10] and is probably due to shielding of mutually repelling charged segments of the polymer chain, which permits the extended chain to assume a more tightly coiled configuration or more cooperative structure.

The decrease in the relative viscosity of aqueous solutions of sodium ribonucleate, especially in the presence of urea, suggests that the nucleic acid initially may be in the form of loose aggregates held together by hydrogen or other bonds. These bonds are then disrupted in the presence of urea. A similar effect of urea upon the viscosity of desoxyribonucleic acid solutions has been observed by Conway.[10]

Additional evidence for structural differences of YRNA in aqueous, dilute salt, and urea solutions is presented in TABLE 2. The absorbancy of an alkaline hydrolyzate of YRNA at 260 mμ is 248. The sodium ribonucleate is therefore moderately hypochromic in water, urea, and KCl-urea solutions, and is more strikingly hypochromic in 0.08 M KCl alone. The specific rotation of sodium ribonucleate solutions in water is strikingly high compared with its constituent nucleotides. KCl (0.08 M) elevated the specific rotation even further. Urea lowered the specific rotation both in the absence and presence of electrolyte.

Hypochromicity appears to be related to the extent of nonrandom orientation of polynucleotide chains and is especially pronounced in multistranded structures made up of complementary chains.[11, 12] It would appear that the sodium ribonucleate assumes a structural configuration more cooperative in the presence of electrolytes than in the presence of electrolytes and urea. A similar interpretation may be made on the basis of the optical rotation of the sodium ribonucleate. The high dextrorotation in the presence of salt and the

TABLE 2

OPTICAL PROPERTIES OF YRNA SOLUTIONS

Solution	$E^{1\%}_{1\text{ cm.}}$	α^{25}_{D}
Water	196	+98°
0.08 M KCl	172	+149°
3.2 M urea	192	+74°
0.08 M KCl + 3.2 M urea	208	+118°
Alkaline hydrolyzate in 0.1 M NaCl, *p*H 7.0	248	−13°

low dextrorotation in the presence of urea probably reflect changes in the sodium ribonucleate molecule similar to the structural changes reported by Doty and Yang[13, 14] for several polypeptides and proteins that are dextrorotatory in helical configuration, but levorotatory in the random coil form. The effects with KCl on YRNA then, are quite consistent, indicating a more cooperative structure as measured by viscosity, absorbancy, and optical rotation. A diagrammatic representation of the configuration of sodium ribonucleate in different solutions is presented in FIGURE 11. This anionic polymer in aqueous solution might first exist partially in the form of aggregates that slowly dissociate to smaller fragments. This process would be accelerated by urea. In the presence of KCl, the aggregates appear to contract to form tightly coiled, cooperative structures, possibly of helical multistranded nature. In the presence of urea, the aggregates appear to degrade into smaller chains or fragments of largely random-coil character. In the presence of urea plus KCl, sodium ribonucleate may be made of single-strand chains in a less cooperative configuration, but still in a contracted form. The enzyme would form smaller oligonucleotides that still may be hydrogen-bonded; urea would then break these bonds and solubilize the products of the reaction.

Part 2

Disulfide bonds and RNase activity. The amperometric technique for sulfhydryl determination was adapted, with modifications, to the measurement of disulfide bonds.[15, 16] The basic principle outlined by Kolthoff and Lingane[17] is

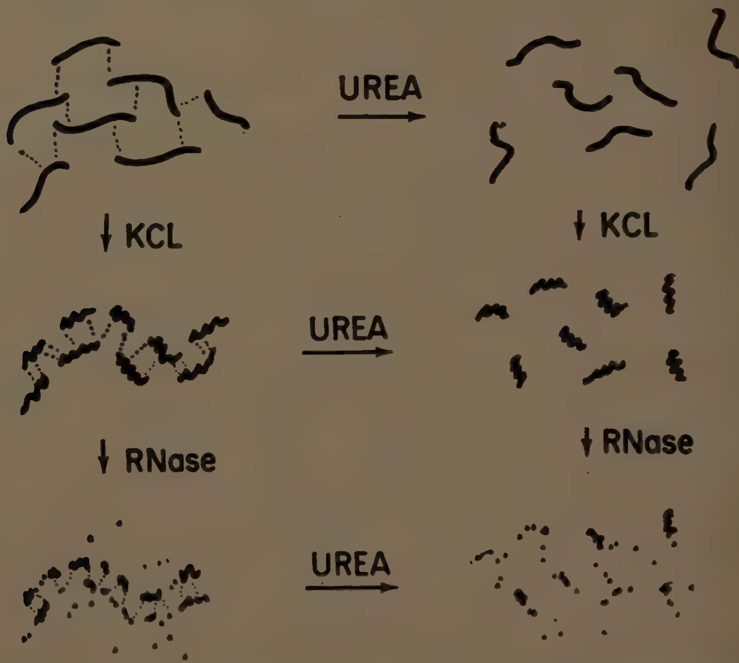

FIGURE 11. Diagrammatic representation of the probable effect of electrolytes, urea, and RNase upon the state of RNA.

the sulfitolysis of the protein disulfide bonds according to the following reactions:

$$R—S—S—R' + SO_3^{=} \rightleftharpoons RS^- + R'—S—SO_3$$

$$RS^- + H^+ \rightleftharpoons RSH$$

$$RSH + Ag^+ \rightleftharpoons R—S—Ag + H^+$$

Thus, 1 mole of —S—S— yields 1 mole of —SH after reaction with Na_2SO_3. The platinum electrode is attached to a galvanometer; the reference electrode is Hg-HgO-saturated $Ba(OH)_2$. The titration vessel contains 0 to 8 M urea, 6×10^{-5} M Versene, and 2 mg. of dialyzed RNase. To this is added, with stirring, 0.1 ml. of a saturated, freshly prepared Na_2SO_3 solution. The total volume is 30 ml. at 37° C. Thirty seconds after the addition of the Na_2SO_3, when the sulfitolysis reaction was complete, aliquots were taken for enzyme assay. The —SH groups were then titrated with 0.001 M $AgNO_3$, after which additional aliquots were taken for assay. During the assay, the aliquots of the enzyme-urea solution were so greatly diluted that the urea could not affect the substrate.

TABLES 3 and 4 show the relationship obtained between the amount of disulfide bonds split and the activity remaining with native RNase and RNase *A*.[18] With no urea present, no —S—S— bonds are split; with increasing concentration of urea, increasing amounts of —S—S— bonds are split to the point where 4 disulfide bonds are broken per mole of RNase in 6 to 8 M urea, at 37° C. These assays were carried out at *p*H 7.3 before we became concerned with the reversibility of the reaction. Cecil and Loening[19] have reported that between

TABLE 3

THE RELATIONSHIP BETWEEN RNASE ACTIVITY AND DISULFIDE BONDS SPLIT

Urea concentration (M)	Moles —S—S— split per mole RNase	Percentages of activity remaining (*p*H 7.3)	Moles —S—S— split per mole RNase	Percentages of activity remaining (*p*H 5.0)	
				Before addition of Ag^+	After addition of Ag^+
0	0	100	0	100	100
4	0.8	88	0.9	100	100
5	2.0	80	2.1	74	53
6	3.9	37	3.1	59	47
6.5	4.0	18	3.5	26	0
8	4.0	0	4.0	0	0

TABLE 4

THE RELATIONSHIP BETWEEN RNASE *A* ACTIVITY AND DISULFIDE BONDS SPLIT

Urea concentration (M)	Moles —S—S— split per mole RNase	Percentages of activity remaining (*p*H 7.3)	Moles —S—S— split per mole RNase	Percentages of activity remaining (*p*H 5.0)	
				Before addition of Ag^+	After addition of Ag^+
0	0	96	0	100	97
3	0.7	87	0.4	92	87
4	0.8	91	0.6	94	95
5	2.1	79	1.9	76	57
6	3.9	34	3.1	33	0
6.5	3.9	9	3.3	0	0
8	4.0	0	4.0	0	0

*p*H 7.2 and 9.0 the thiol groups formed in the reaction with insulin begin to ionize and the reaction becomes reversible. The reaction is largely reversible at *p*H 9, and not at 7.2. Subsequent assays were carried out at *p*H 5.0 within 1 min. after the sulfitolysis reaction had gone to completion. Additional aliquots were taken for assay after titration with $AgNO_3$. With almost 1 —S—S— bond split per mole of RNase, activity was still essentially complete; with approximately 2 —S—S— bridges split per mole, enzyme activity still remained high; with 3.9 disulfide links cleaved in 6 M urea, some activity still remained; and with 4.0 —S—S— bonds split per mole of RNase in 8 M urea, inactivation was complete.

Assays carried out at *p*H 7.3 and 5.0 gave very similar results indicating that

the sulfitolysis reaction was not reversible under the conditions of these experiments. The presence of Ag^{+}, which was somewhat inhibitory to RNase activity, probably accounted for the somewhat reduced activity remaining after titration with $AgNO_3$. Plots of the moles of —S—S— split per mole of RNase relative to the percentage of activity remaining (FIGURE 12) for both native and the *A* fraction of RNase gave 2 curves that were almost identical. These results are in general agreement with the published results of Sela *et al.*,[20] determined by a different method. The results indicate that probably 1 and possibly 2 —S—S— links may not be required for the catalytic activity of this enzyme. The fact that some activity still remained with 3.9 —S—S— bonds cleaved, in *6 M urea*, whereas no activity remained when all 4 cystine bridges were broken in *8 M urea* would indicate that the —S—S— bridges served to

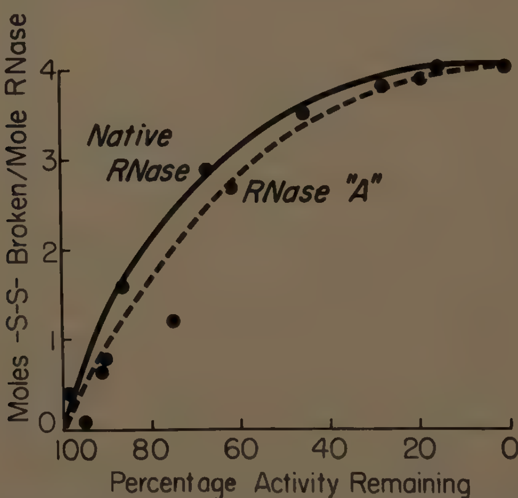

FIGURE 12. The effect of breaking disulfide bonds on the activity of native RNase and RNase *A* as measured spectrophotometrically.

maintain the secondary structure of the enzyme, and that this structural integrity was essential for the activity of the enzyme. Therefore, experiments were undertaken to determine the relationship of the secondary structure of RNase to its enzymatic activity.

The relationship of the secondary structure of RNase to its enzymatic activity. Doty *et al.*[21] recently have shown that, when RNase is dissolved in 2-chloroethanol, it undergoes transition to a new configuration, characterized by a high content of helical polypeptide structures. Weber and Tanford,[22] working with RNase, found that at *p*H 4.5 to 5.0 and ionic strength 0.02 to 0.2 a solution containing 31 parts water to 25 parts chloroethanol (by volume basis) gives maximum uncoiling in this system. When the enzyme is recovered from this chloroethanol solution and redissolved in water, it regains its full activity. However, we wished to assay RNase in its uncoiled form in this system. FIGURE 13 presents the activity of the enzyme in water and in chloroethanol-water solutions. RNase in its uncoiled state, in chloroethanol-water solution, is

about 50 per cent less active than it is in water solution alone, suggesting that the secondary structure of this molecule is associated with enzyme activity. However, the inhibition might be simply a reflection of a solvent effect.

Fortunately, by working in a water medium, we have been able to circumvent objections based on this effect. Tanford and Weber[23] have carried out some exploratory experiments (at *p*H 4.7 to 5.0 and ionic strength 0.02 to 0.2 μ) re-

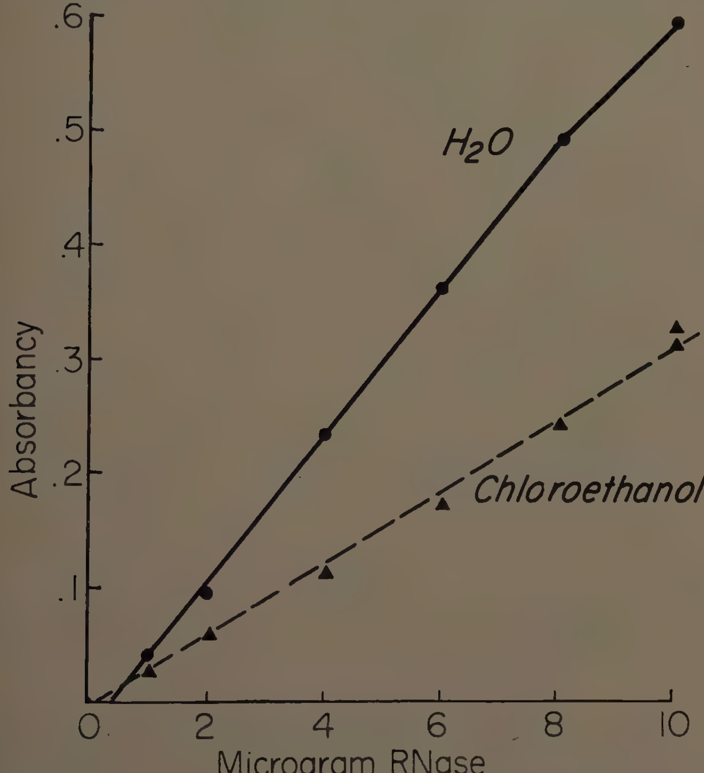

FIGURE 13. Spectrophotometric assay of RNase activity in aqueous or chloroethanol systems. Conditions: 0.01 ml. of RNase solution was incubated with 2 ml. of 0.25 per cent yeast ribonucleate in 0.05 M acetate, with the *p*H 5.0, in water or chloroethanol-water in a 25:31 ratio.

lating the intrinsic viscosity of RNase to the temperature. They find that the intrinsic viscosity of the enzyme starts to increase at 50 to 55° C. and reaches a maximum at 70° C., the reaction being reversible. What we have done, then, is simply to assay RNase at *p*H 5.0 and to determine its initial velocities at various temperatures (FIGURE 14). The initial velocity increases sharply from 30 to 50° C., more slowly to 60° and 65° C., starts falling at 70° C., and shows little or no activity at 75° and 80° C., respectively. Fom the initial velocities at these temperatures we have calculated the slopes or velocity constants of RNase at various temperatures (TABLE 5). The ratio of the velocity constant

at 40° C. to that at 30° C. is 2.87, almost a threefold increase in activity with a 10° rise in temperature. Almost the same increase is obtained between 40° and 50° C. Between 50° and 60° C., where the intrinsic viscosity of RNase

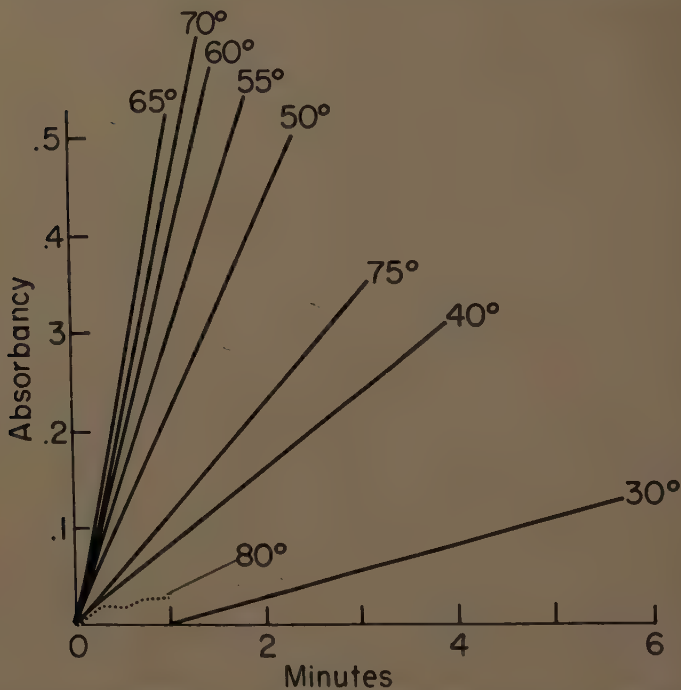

FIGURE 14. The influence of temperature upon RNase activity as measured spectrophotometrically. Conditions: 4 μg. of ribonuclease incubated with 2 ml. of 0.5 per cent yeast ribonucleate in 0.1 M acetate, *p*H 5.0.

TABLE 5

VELOCITY CONSTANTS OF RNASE AT VARIOUS TEMPERATURES

Temperature °C	Velocity K	Ratio K_2/K_1 (Q_{10})
30	0.271	
40	0.778	2.87
50	2.130	2.74
55	2.91	
60	3.714	1.74
65	5.00	1.72
70	4.38	1.18
75	1.14	0.23

starts to increase,[23] there is a sharp drop in the ratio of the velocity constants. The same (lower) ratio is obtained between 55° and 65° C. In FIGURE 15 we have plotted the log of K, the velocity constant, relative to $1/T$ on the Kelvin scale. The three points obtained at 30°, 40°, and 50° C. fall on the lower straight line. From the slope of this line we have calculated the activation

energy of the reaction to be approximately 20,290 cal. The other 3 points were obtained at 55°, 60°, and 65° C., with the break appearing between 50° and 55° C., precisely where the enzyme molecule starts to unravel. It should be noted that, in this spectrophotometric assay measuring the liberation of acid-soluble oligonucleotides by RNase, the blank values were exactly the same at all temperatures from 30° to 80° C., indicating the temperature stability of the substrate as far as the assay is concerned. It appears, therefore, that the secondary or coiled structure of the RNase molecule is essential for its activity.

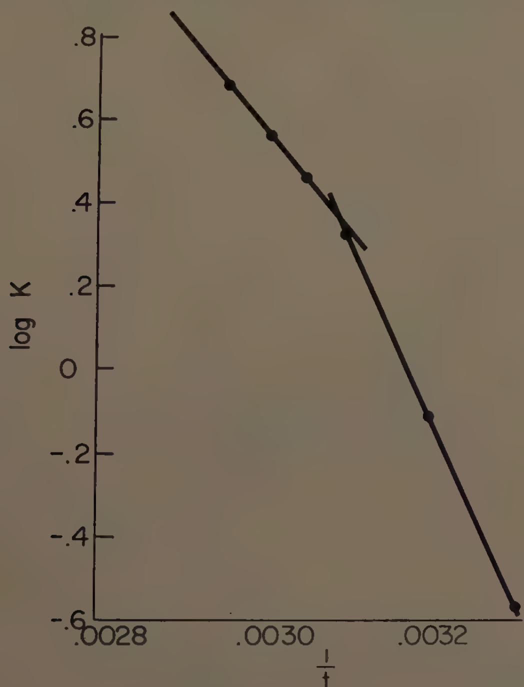

FIGURE 15. An Arrhenius plot relating the velocity constants (K) of RNase activity and absolute temperature (T).

Partially hydrolyzed RNase with enzymatic activity. Studies in our laboratory on the mechanism of action of enzymes started with models, involving the Mn^{++}-catalyzed oxidation of α-ketoglutarate in the absence of enzyme. This reaction was accelerated by amino acids. Threonine proved to be the most effective, the —NH_2, —OH, and —COOH groups all being necessary for activity.[24, 25] Since, in this instance, threonine owed its activity to the presence of its three polar groups, an analogy was drawn with enzymes in which the whole, intact protein might not be necessary for proteolytic activity; the specificity and activity of the enzyme might be due to an "active center" containing the correct spatial configuration of active groups on the surface of the protein, enabling it to react with the substrate.

Crystalline RNase was selected as a suitable starting material, since it is stable, has a relatively simple structure, requires no coenzyme or metal ion, is

easily obtainable in electrophoretically homogeneous form, is assayed fairly readily, and is inexpensive. The RNase was digested with crystalline preparations of carboxypeptidase, resulting in the liberation of a number of different amino acids. Enzymatic assay of the digested RNase showed no decrease in activity.[26-28] Controls containing RNase or carboxypeptidase alone produced no amino acids under identical conditions. Chromatography of the digests on Amberlite IRC-50 (XE-64)[18] did not separate active, digested molecules from native enzyme molecules, but paper chromatography did.[27] After descending chromatography in secondary butanol-water (3:1) at 5° C. for 16 hours, native RNase (alone or with carboxypeptidase at zero time) did not migrate and could

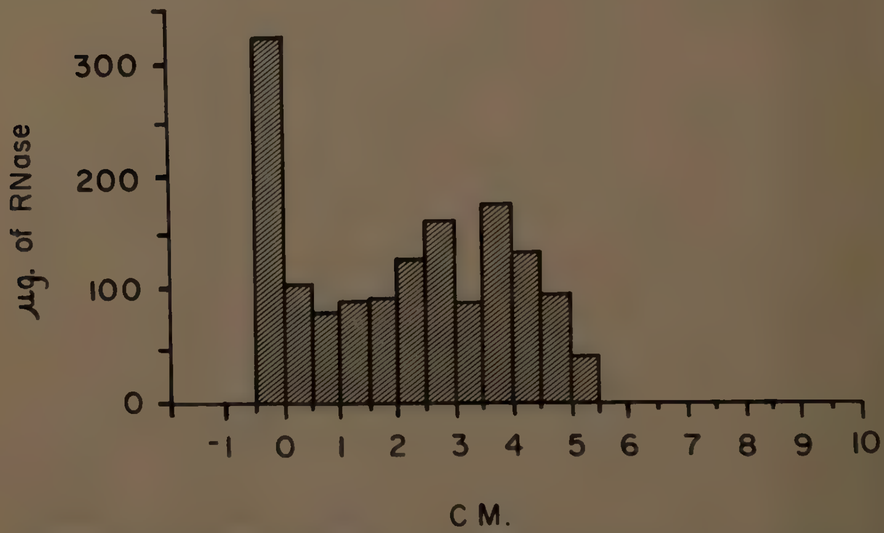

FIGURE 16. Migration of digested RNase in paper chromatography. RNase (84 mg.) and carboxypeptidase (3.3 mg.) in 1 ml. of water were incubated at *p*H 8.1 for 34 hours at 25° C. and the digest was chromatographed with secondary butanol-water (3:1) at 5° C. on Whatman No. 1 filter paper. The bars represent the RNase activity on various sections of the paper chromatogram. Undigested RNase was confined to the region of the first 2 bars.

be recovered quantitatively at the origin; none was ever detected below this point. Digested RNase, however, did migrate down the paper (FIGURE 16).

Since RNase catalyzes 2 reactions, an intramolecular transphosphorylation followed by hydrolysis,[29, 30] RNase was partially inactivated by hydrolysis with carboxypeptidase, and the resulting solutions were analyzed for enzymatic activity by 3 different methods (TABLE 6). The spectrophotometric assay has already been described in *Part 1*; it is essentially a modification of published procedures[2-4] and measures the production of acid-soluble oligonucleotides from sodium ribonucleate. The second assay listed in TABLE 6 measures the hydrolysis of 2′,3′-cyclic cytidylic acid to 3′-cytidylic acid; the procedure, using paper chromatography, was similar to that of Davis and Allen[31] and Heppel and Whitfield.[32] The third assay was the manometric assay of Bain and Rusch,[33] which depends on the liberation of acid groups from sodium ribonucleate in a bicarbonate medium and is a measure of the over-all reaction. It appears that

different assay methods give identical results with partially inactivated RNase (TABLE 6). Under the conditions of these experiments, the 2 catalytic activities of the enzyme are destroyed at the same rate. There is no indication here of 2 active centers in the molecule.

Different amounts and different lots of commercial, 5 times recrystallized carboxypeptidase were used in the 2 experiments cited in TABLE 6. In Experiment 1, a smaller amount of carboxypeptidase than that used in Experiment 2 produced a greater amount of inactivation in a shorter time, with the liberation of more histidine. This points to different properties of the 2 lots of this digestive enzyme. This is further borne out by the elution patterns of the native and digested RNase on Amberlite IRC-50 (XE-64) shown in FIGURE 17. The elution pattern of 1 mg. of native ribonuclease is shown in FIGURE 17*a*; that of the 114-hour digest of 1 mg. of RNase with the less effective carboxypeptidase,

TABLE 6

ASSAY OF DIGESTED RNASE BY DIFFERENT METHODS

Experiment No.*	Carboxypeptidase lot No.†	Hours of digestion	Percentages of activity: Spectrophotometric assay	Percentages of activity: Hydrolysis of cyclic nucleotide	Manometric assay	Moles of histidine liberated per mole RNase
1	C-2802	84	51	49	53	3.1
2	D-2803	48	83	89	80	
		86	76	76		
		114	69	64		1.3

* Experiment 1: 42 mg. ribonuclease plus 13.2 mg. carboxypeptidase; molar ratio 15:1. Experiment 2: 84 mg. ribonuclease plus 20.4 mg. carboxypeptidase; molar ratio 10:1. Conditions: *p*H 7.5 to 8.1; 25° C.; 2 ml. total volume.

† Pentex, Inc., Kankakee, Ill.

which liberated 1.3 moles of histidine per mole of RNase is shown in FIGURE 17*b*, where the smaller *B* peak has flattened, the *A* peak has shortened, and inactive protein has appeared. FIGURE 17*c* shows the elution pattern of a similar amount of digested RNase, where greater inactivation was obtained with the liberation of 3.1 moles of histidine per mole of RNase. Here, the small peak has disappeared entirely, the *A* peak is considerably shortened, and a considerable amount of inactive protein has appeared. Unfortunately, these patterns clearly demonstrate different properties for the 2 different lots of carboxypeptidase, which can probably be explained by the varying content of contaminating proteolytic enzymes. In subsequent experiments, therefore, a different proteolytic enzyme has been employed to digest RNase in attempts to obtain an active fragment of this latter enzyme.

A fungal proteinase* has been used with interesting results. FIGURE 18 presents the elution patterns of this proteinase and of RNase on 0.9 × 60-cm. Amberlite IRC-50 columns. On this column, which is twice the length of the ones previously used, this proteinase appears to be a fairly homogeneous ma-

* Purchased from Nutritional Biochemicals, Cleveland, Ohio.

terial, coming out ahead of the RNase. Proteinase gives only about one tenth the color intensity of RNase, using the protein method of Lowry *et al.*[34] whereas, with the ninhydrin method, almost the reverse is found.

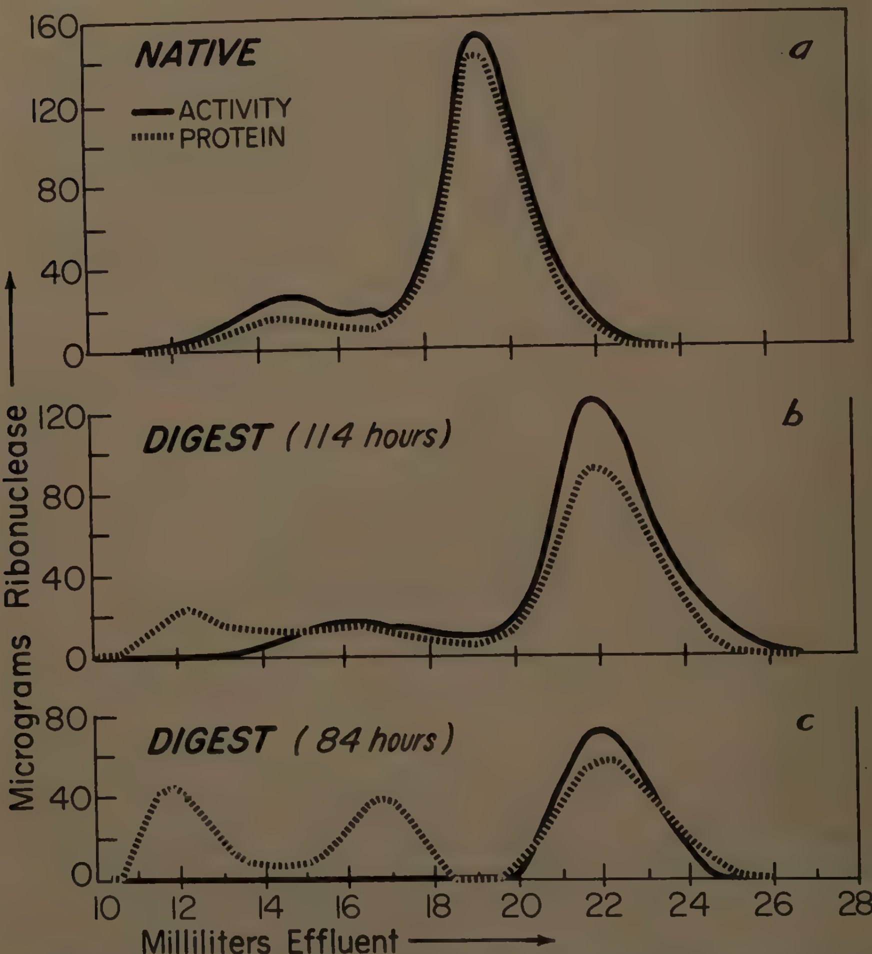

FIGURE 17. Elution patterns of carboxypeptidase-digested RNase on Amberlite IRC-50 (XE-64). (*a*) Pattern of 1 mg. of native RNase. (*b*) Pattern of the 114-hour digest of 1 mg. of RNase with the less effective carboxypeptidase. (*c*) Pattern of a similar amount of digested RNase. The major peak of activity, which was eluted last, corresponds to RNase *A*, while the smaller active fraction immediately preceding corresponds to RNase *B* of Hirs *et al.*[18]

FIGURE 19 illustrates the rate of digestion of RNase with proteinase, at *p*H 7.0, as followed in the *p*Hstat. In the presence of proteinase alone, there is a very small liberation of hydrogen ions during a 4-hour period. On the addition of RNase to the proteinase (the ratio of RNase to proteinase being 2:1 on a weight basis) there is a fairly rapid uptake of NaOH to maintain the *p*H at 7.0. If aliquots are taken from this solution for assay at various periods of time, we

find that the loss of activity parallels the rate of digestion. The lower set of curves is obtained with a ratio of RNase to proteinase of 4:1. The rate of alkali uptake is a little slower but, again, activity decreases as digestion proceeds.

After 4 and 5 hours of digestion, aliquots were withdrawn from the digestion mixture and chromatographed on 60-cm. Amberlite columns.

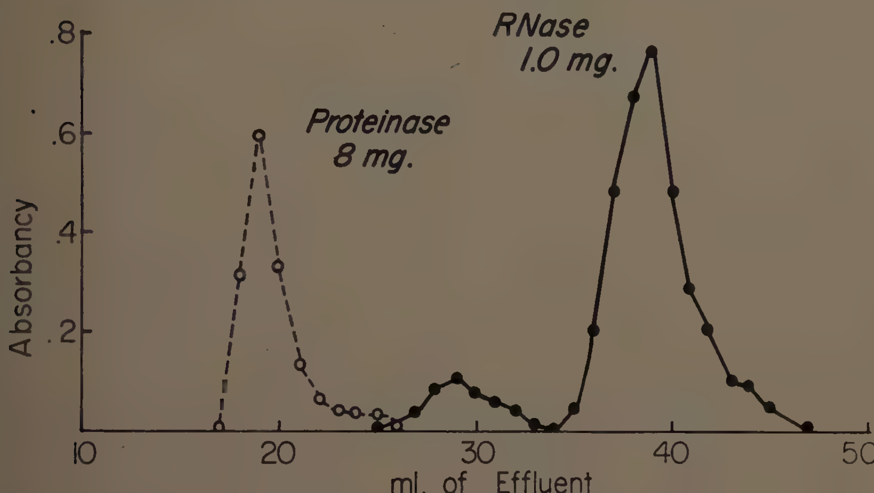

FIGURE 18. Elution pattern of fungal proteinase and RNase.

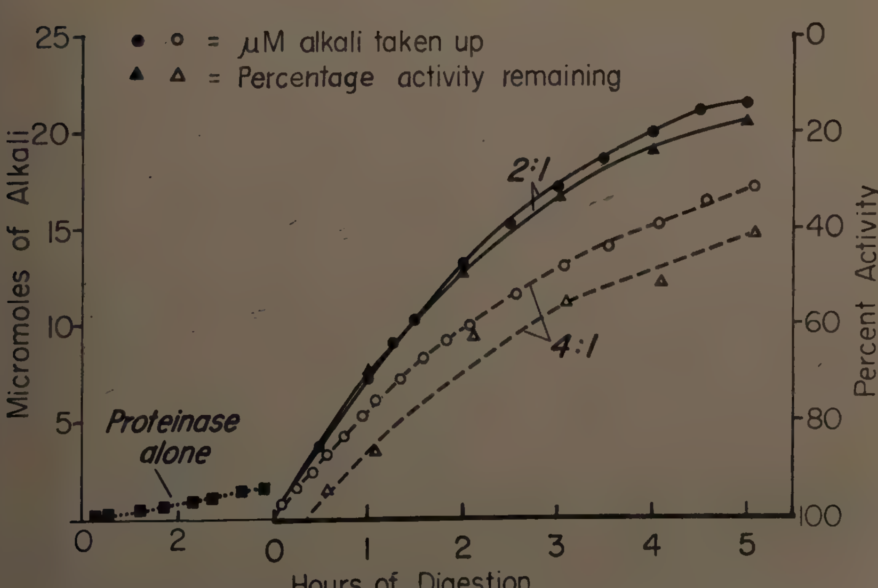

FIGURE 19. Rate of digestion and inactivation of RNase by fungal proteinase.

FIGURE 20 shows the elution patterns of 1 mg. of native and of digested RNase. After 4 hours of digestion, about 25 per cent of the original RNase activity remains, but the portion of the enzyme that is still active has a typical elution pattern: a tall peak, *A*, a short peak, *B*, plus a third, enzymatically active, smaller peak. After 5 hours of digestion, the height of peak *A* has decreased, peak *B* appears to have increased, and the third peak is still in evidence. The fourth elution pattern (FIGURE 20, *lower right*) was obtained in another experiment under the same conditions as described above for the 4-hour digest (*upper right*), and gave almost identical results. Again, we have peaks *A* and *B* of native RNase, plus the appearance of a third, enzymatically active one.

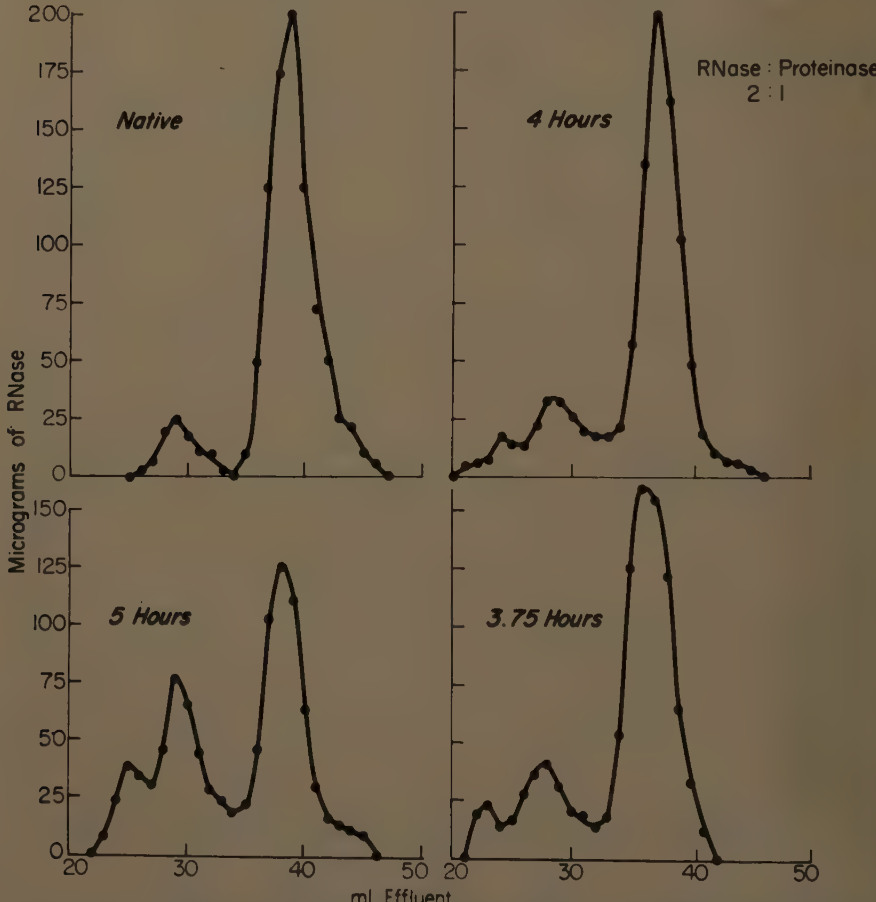

FIGURE 20. Elution patterns of partially digested RNase (RNase-proteinase, 2:1) on Amberlite IRC-50 (XE-64). Vertical axis represents RNase activity. The *A* fraction is the slowest moving peak, the *B* fraction is that immediately preceding it, while the *C* fraction is the most rapidly eluted, enzymatically active fraction.

With less proteinase present: that is, with a RNase to proteinase ratio of 4:1, the elution patterns of RNase obtained after digestion for 4.25, 5, and 11.3 hours are a little different (FIGURE 21). In the elution patterns of these digests the *A* peak is diminished and, again, enzymatically active RNase material makes its appearance.

The active center of RNase. The inactivation of RNase by ultraviolet light has been interpreted by McLaren *et al.*[35] as indicating that the primary process involved in the inactivation was a modification of aromatic residues of the

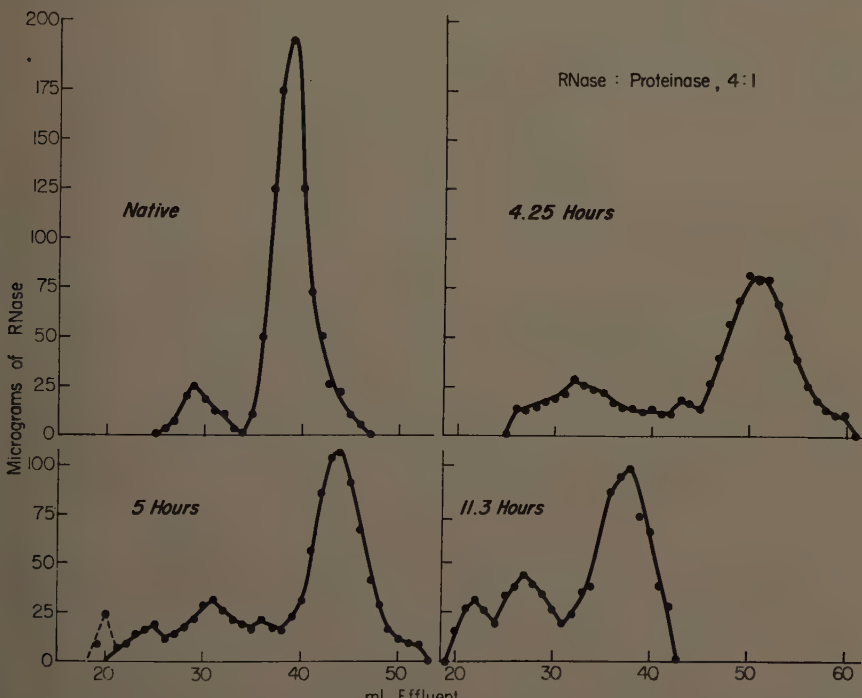

FIGURE 21. Elution patterns of partially digested RNase (RNase-proteinase, 4:1) on Amberlite IRC-50 (XE-64). The axes and designations of peaks are the same as in FIGURE 20.

enzyme. This is in agreement with the subsequent important findings of Weil and Seibles[36] who subjected RNase to photo-oxidation in the presence of methylene blue. These investigators found that, with the oxidation of 0.5 mole of each molecule of a histidine residue, 52 per cent inactivation occurred; oxidation of 1 mole resulted in a 74 per cent decrease in enzyme activity; and complete inactivation was obtained with the photo-oxidation of 3 moles of histidine of the total of 4 present in the molecule.

Kalnitsky and Rogers[27, 28] demonstrated that a number of free amino acids may be liberated from RNase by digestion with commercial crystalline carboxypeptidase, with no decrease in ribonuclease activity. Of particular interest in these experiments are those amino acids that occur infrequently in the RNase molecule. They found that with moderate digestion, 1 of the 4 histidine resi-

dues and 1 of the 3 isoleucine residues could be split off without affecting the activity; some loss in activity was observed when the digestion had proceeded to the extent that additional amounts of histidine and isoleucine were split off. Partial activity remained even when almost all of the 2 leucine residues were digested away from RNase.

The approximate location of these residues in the RNase molecule is known from the partial structural formula for oxidized RNase made available by the excellent work of Hirs *et al.*[37] The 20-residue peptide that constitutes the C-terminal end of RNase contains 2 isoleucine residues that lie close together, plus 2 histidine, but no leucine residues. Since the liberation of more than 1 isoleucine and more than 1 histidine appears to be associated with inactivation of the enzyme as assayed in these experiments, it is possible that the active center may be situated relatively close to the C-terminal end and may be contained in less than 20 per cent of the original enzyme molecule.[28] This would be in agreement with Anfinsen's finding that the loss of the C-terminal tetrapeptide by digestion with pepsin causes inactivation of RNase.[38]

The evidence linking histidine and the C-terminal portion of RNase with its active center is supported by the work of still another group of investigators (Barnard and Stein[39]) using a different approach. Bromoacetic acid introduces a stable carboxymethyl group into proteins[40] and reacts with histidine in RNase, inhibiting the enzyme. The reaction with bromoacetic acid is prevented by the presence of the competitive inhibitor, cytidylic acid. It appears that a specific reaction occurs at 1 histidine residue in the active center of the enzyme; the histidine concerned is unequivocally identified (using C^{14}-labeled bromoacetic acid and degradation with chymotrypsin[37]) as that nearest the C-terminal end of the RNase molecule,[39] which contained the major part of the C^{14}-label.

However, there is more to be said. Richards,[41] as a result of digesting RNase with subtilisin, has split off a peptide containing 20 amino acids from the N-terminal portion of RNase without inactivating the enzyme. Separation of the peptide from the remainder of the protein with trichloroacetic acid results in complete inactivation; activity can be completely regenerated simply by recombination under certain conditions. The fact that splitting of peptide bond No. 20 did not result in inactivation of RNase, and removal of the peptide did, indicates that association between the peptide and the remainder of the protein in some form of secondary structure is necessary for the catalytic activity of RNase. In addition, Richards' peptide contains 1 histidine residue that can be inactivated by photo-oxidation.[41] Therefore, it is indeed tempting to speculate, as Richards has done, that perhaps 2 of the 4 histidine residues are essential for RNase activity, 1 near the C-terminal end of the molecule, the other near the N-terminal end, the 2 being linked through the secondary structure of the enzyme protein. Elevated temperatures may be as effective in altering the secondary structure and separating these 2 sites as Richards' procedure of digestion and precipitation with trichloroacetic acid. However, as previously stated,[28] unequivocal location of the "active center" must await its isolation and characterization.

With the recognition that crystalline enzyme preparations are not necessarily pure, it becomes important that only highly purified preparations of RNase be used for degradation studies. We have found that reliable measure-

ments of the disulfide content of RNase can be made only after thorough dialysis to remove ammonium salts, and Tanford (personal communication) has observed that appreciable quantities of inorganic salts contaminate some commercial preparations. Moreover, although significant quantities of free amino acids may be released by treatment of RNase by carboxypeptidase with no loss of enzymatic activity and, although the digested ribonuclease exhibits an altered chromatographic pattern, there is no assurance that part of the free amino acids liberated do not arise from small amounts of impurities. For example, Uziel *et al.*[42] have reported that purified RNase is not attacked by trypsin as readily as is commercial RNase. It is apparent, therefore, that RNase *A* should be employed in further degradation studies. By using better assay procedures, purified enzymes, and unambiguous procedures for isolation and characterization of active fragments, it should be possible to work out some additional features of the active center of RNase.

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Discussion

A. A. Hakim (*Miami Heart Institute, Miami Beach, Fla.*): Isolation of homogeneous stable ribonucleic acid (RNA) remains one of the missing factors necessary for the understanding of ribonuclease specific activity. Bourdet and Mandel[1] showed that autolysis, if permitted to proceed at 50° C. for 10 min., destroyed 93 per cent of the nucleic acids originally present, which indicates that the heterogeneity of RNA, as well as their instability, could be due to enzymic degradation in the process of isolation, and to contamination of the isolated acid with minute acids.

The hydrolysis of yeast RNA by pancreatic ribonuclease could be taken as a 2-step reaction. The first step involves the formation of nucleoside 2′,3′-phosphate whereas, in the second step, hydrolysis of the cyclic phosphate to 3′-nucleotide occurs.

Reports on enzymic heterogeneity of crystalline ribonuclease[2, 3] showed that ribonuclease *B* liberates guanylic acid from ribonucleic acid and catalyzes synthesis of certain dinucleoside phosphates; it is similar to ribonuclease *A* and to the crystalline ribonuclease and hydrolyzes RNA, liberating mononucleotides. It also hydrolyzes cyclic guanylic acid to guanosine 3′-phosphate, whereas ribonuclease *A* does not. These specific activities could not be observed on unstable heterogeneous nucleic acid preparations, where the enzymes have already acted.

One yeast RNA sample each was prepared according to 4 procedures; those of (1) Kay and Dounce,[4] (2) Crestfield *et al.*,[5] (3) Hakim,[6] and (4) Grinnan and Mosher.[7] Two other samples were obtained commercially, dialyzed for 48 hours at 0° C., and lyophilized.

Aliquots of each preparation were dissolved in sterile distilled water, adjusted to *p*H 7.0, and kept at 0° C. At 48-hour intervals for 3 months, 0.5 ml. of each was subjected to paper chromatography. All the above nucleic acid preparations showed the appearance of cyclic mononucleotides, dinucleotides, and polynucleotides in 4 weeks, except the sample prepared by Procedure 3, which failed to show any cyclic mono- or dinucleotides; even at the end of 3 months, polynucleotides were found only in traces.

Each of the above nucleic acid samples was dissolved in sterile distilled water. The solution of each of the samples was divided into 6 aliquots, adjusted to *p*H 4.00, 5.00, 6.00, 7.32, 8.00, and 9.00, respectively. At weekly intervals for 2 months, 0.100-ml. aliquots were removed from each and analyzed by paper chromatography. Cyclic mononucleotides appeared at *p*H 7.32 and alkaline *p*H much faster than at lower *p*H. Figure 1 illustrates a chromatogram of a sample prepared by Procedure 2, after 4 weeks at 0° C.

The stability of the sample prepared by Procedure 3 made it more available for studies on ribonuclease specificity. Chromatograms of the digests of this

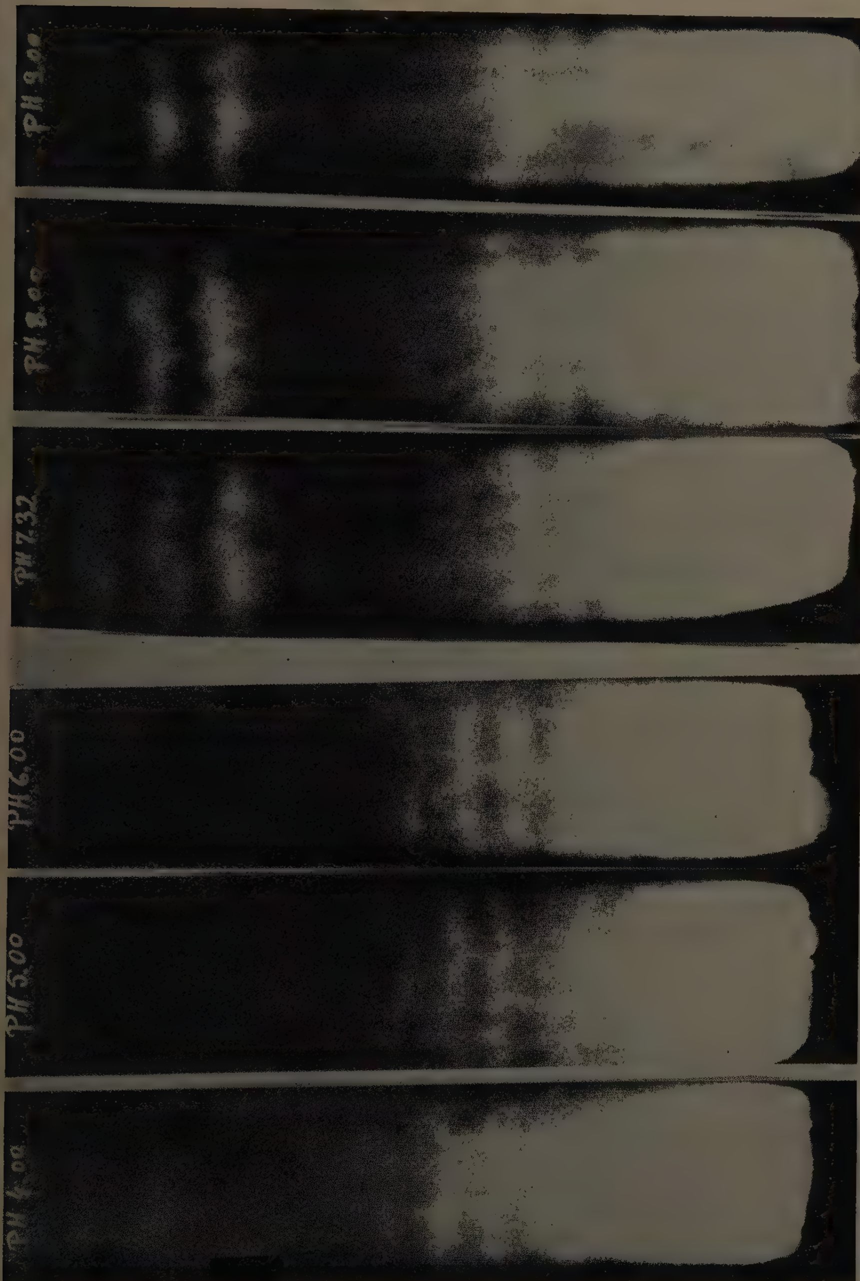

FIGURE 1. Chromatograms of RNA sample prepared according to Procedure 2 (see text) after 4 weeks at 0° C. and at *p*H shown.

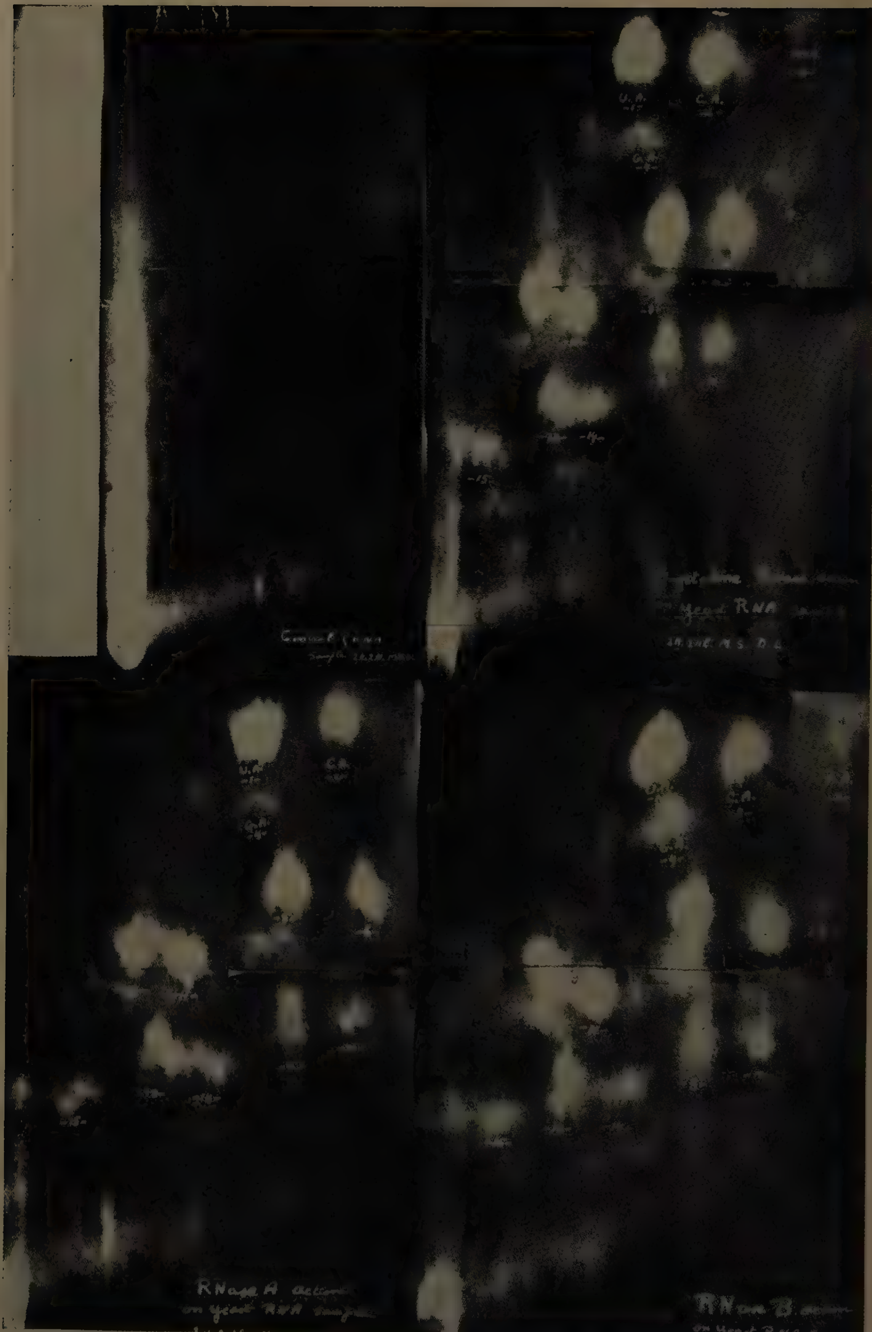

FIGURE 2. Chromatograms of digests of RNA sample prepared by Procedure 3 (see text), with crystalline ribonuclease, ribonuclease *A* and *B*, and control. Letters designate acids, as follows: U.A., uridylic acid; C.A., cytidylic acid; A.A., adenylic acid; and G.A., guanylic acid.

RNA with each of the enzymes, with crystalline ribonuclease, and with ribonuclease *A* or ribonuclease *B* are presented in FIGURE 2. The enzymic hydrolysis was carried out by mixing 0.5 ml. of phosphate buffer (containing 10 mg. of RNA) with 0.25 ml. of a solution of 0.2 mg. of each of the ribonuclease samples in 0.2 M phosphate buffer, both at *p*H 7.2 and incubated at 37° C. for 48 hours. The chromatograms were developed in ammonia-isobutyric acid for the short dimension, and in tertiary amyl alcohol-formic acid-water (3:3:1) for the second dimension. The identities of the mono-, di-, tri-, and tetranucleotides located on the chromatogram were described in earlier communication.[8] As shown in FIGURE 2, ribonuclease *B* liberates more guanylic acid terminating di- and trinucleotides than crystalline ribonuclease or ribonuclease *A*.

In summary, we may conclude that most yeast RNA preparations are unstable in aqueous solutions, since cyclic mononucleotides were produced in aged aqueous solution. The instability of these preparations is due to the contamination of the nucleic acid preparations with traces of ribonuclease.

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FRED KARUSH (*School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): The recovery of enzymatic activity after full reduction of ribonuclease, described by F. H. White, is of considerable interest because of its bearing on the problem of the pairing of SH groups leading to the formation of disulfide. The evidence he presents favors the view I hold regarding the pairing process. For reasons given elsewhere,[1] I believe that the genesis of proteins involves the formation of polypeptide chains in more or less extended form. For those proteins that contain disulfide linkages, this view implies that their precursors exist in the reduced form—that is, that the chain initially contains cysteine residues. The specificity of the subsequent pairing of the SH groups, I suggest, is governed not by enzymatic intervention, but by the sequence of the residues in the chain. This determination could be effected by the energetic advantage gained by particular SH pairings resulting from attractive interactions between side chains in the vicinity of the cysteine residues. The grounds for rejecting enzymatic participation are: (1) there is no energetic need for it, since the formation of disulfides from sulfhydryl groups can take place under physiological conditions without an enzyme, and (2) it appears unlikely that an enzyme could achieve the geometric proximity to the multiple pairs of cysteine residues which would be required if it were to exercise a directing role.

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Part II. Intracelllular Ribonucleases

STUDIES ON THE DISTRIBUTION OF INTRACELLULAR RIBONUCLEASES*

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Introduction

Most of the studies on ribonucleases (RNases)—especially the pancreatic crystalline RNase—were aimed at the elucidation of their mode of action on ribonucleic acid (RNA). From these studies we know that these enzymes depolymerize RNA, with the liberation of various mononucleotides and polynucleotides. But the physiological role of cellular RNases is almost entirely unknown. We have carried out studies on tissue RNases in animals under various physiological and pathological conditions, and the purpose of this report is to review the results.

We have been interested in nucleodepolymerases since 1949, and our interest was enhanced when it was first reported in 1954 by Roth[1] and by us[2] that rat liver contained an RNase showing 2 peaks of activity, 1 in the acid and the other in the alkaline range of *p*H. These observations were made using different methods of assay. The properties of the enzyme(s) at the 2 *p*Hs were different[2,3] and suggested the presence in rat liver of 2 different RNase components; this has been confirmed, as will be shown later. The terms acid and alkaline RNase will be used thus throughout this report. In the first part we shall examine the distribution of acid and alkaline RNases in various tissues, and the intracellular distribution of these enzymes in some of them. In the second part, the levels and intracellular distributions of RNases in the livers of rats under various physiological and pathological conditions will be discussed. A third section deals with some properties of the isolated liver RNases.

Studies on Normal Tissue Ribonuclease

The assay procedure used in this work is that of Schneider and Hogeboom,[4] with slight modifications.[2] This method is based on the determination of the optical densities of the acid-soluble material liberated in the incubation mixture by the action of RNase on RNA. The activity was expressed in arbitrary units that represent the optical density corresponding to the acid-soluble material liberated from RNA per gram of liver pulp during an incubation of 30 min. at 37° C. at a final *p*H of 5.8 or 8.2. Some fundamental kinetic studies have been carried out to establish accurately this assay procedure for routine measurements. The optimal amount of substrate, as well as proportionality between enzyme activity and time of incubation or amount of tissue were determined. The *p*H-activity curve (FIGURE 1) revealed 2 peaks of activity, 1 at *p*H 5.8 and the other at *p*H 8.2. Similar optimal *p*Hs of activity were

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observed by Roth[1] for rat liver homogenates. FIGURE 2 shows the *p*H-activity curve for brain homogenate. The presence of 2 peaks, 1 at *p*H 5.5 and the other at *p*H 8.0 was similarly observed. Roth found the same 2 peaks of activity in kidney homogenate.[1] Maver and Greco found only 1 peak of activity in spleen homogenate,[5] but Hilmoe and Heppel distinguished 3 RNase activities in this tissue.[6]

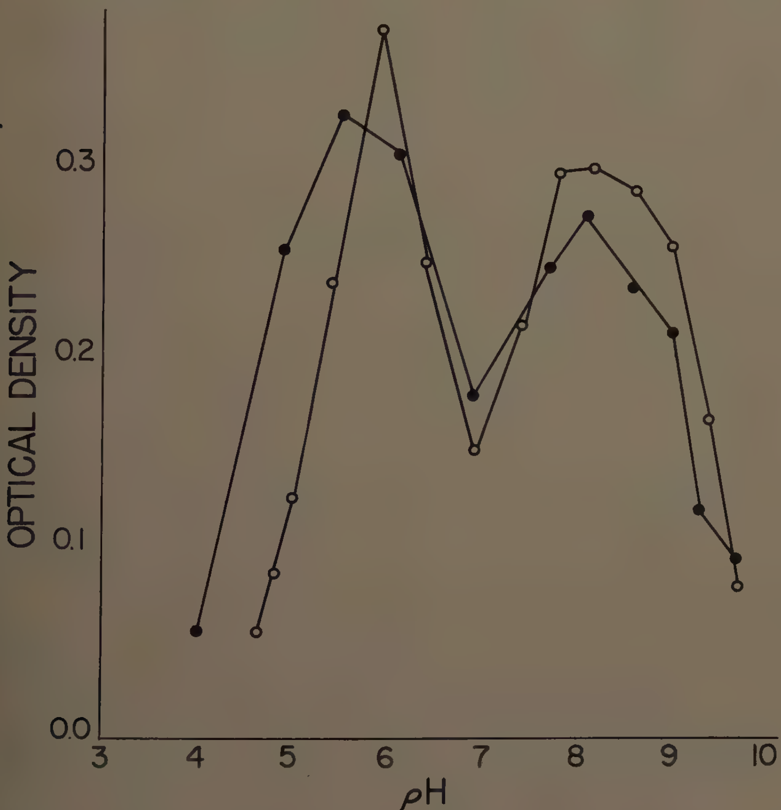

FIGURE 1. The *p*H-activity curve of RNase of rat liver homogenate. *Closed circles* indicate activity in acetate-borate-cacodylate buffer; *open circles*, activity in phosphate-borate-cacodylate buffer.

These observations prompted us to reinvestigate the distribution of RNases in various tissues, as well as their intracellular distribution in cell fractions isolated by differential centrifugation.[7] TABLE 1 shows the distribution of acid and alkaline RNase activities in mouse tissue. Activity at both *p*Hs was obtained for the 4 tissues examined, and the activity of the alkaline enzyme was greater than that of the acid one, as can be seen by the ratio of alkaline to acid RNase activity. However, the level of activity varied greatly from tissue to tissue. Acid RNase activity was lowest in liver and highest in intestinal mucosa; alkaline RNase activity was 4 times lower in liver than in intestinal mucosa.

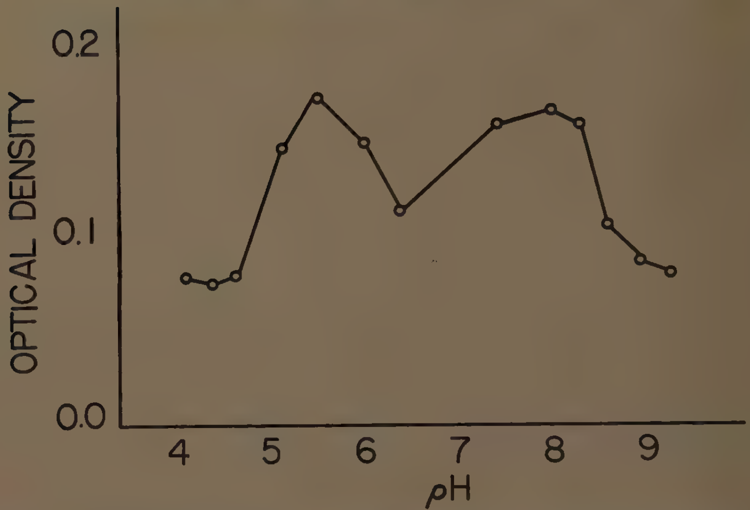

FIGURE 2. The *p*H-activity curve of RNase of rat brain homogenate; the buffer is acetate-borate-cacodylate.

TABLE 1
ACID AND ALKALINE RNASE ACTIVITIES IN MOUSE TISSUES

Tissue*	Acid	Alkaline	Alkaline-acid ratio
Intestinal mucosa (4)	1200	6560	5.4
Spleen (8)	884	3162	3.5
Kidney cortex (4)	955	2288	2.4
Liver (25)	592	1565	2.6

* Tissue homogenates were prepared as described in Allard *et al.*,[8] and the enzymatic activity determined as described in de Lamirande *et al.*,[2] except that a 1 per cent homogenate was used for kidney and intestine, and a 5 per cent homogenate for spleen. The figures in parentheses represent the number of assays.

TABLE 2
ACID AND ALKALINE RNASE ACTIVITIES IN RAT TISSUES

Tissue*	Acid	Alkaline	Alkaline-acid ratio
Intestinal mucosa (4)	1475	9390	6.3
Spleen (3)	900	3887	4.3
Thymus (3)	477	1693	3.6
Kidney cortex (4)	2282	6265	2.7
Liver (7)	338	786	2.3
Heart (3)	125	281	2.2
Brain cortex (3)	110	241	2.2

* See footnote, TABLE 1.

The levels of alkaline RNase activity in these tissues suggest a correlation with their respective rates of cellular division; this will be discussed below.

TABLE 2 shows the distribution of acid and alkaline RNase activity in seven tissues of the rat; intestinal mucosa, spleen, thymus, kidney cortex, liver, heart, and brain. The activity of the alkaline RNase in these tissues was greater than that observed for the acid enzyme in mouse tissue. The enzymatic activities of intestinal mucosa and spleen of the rat were the same as those of the corresponding tissues of the mouse. However, there seemed to be a species difference for kidney and liver. The levels in rat kidney were about three times those of mouse kidney, whereas the activities observed in rat liver were about half those of mouse liver.

The range of RNase activity was much larger in rat tissue than in mouse tissue. Brain cortex showed twenty times less acid RNase activity than kidney, and forty times less alkaline RNase activity than intestinal mucosa. A correlation between the rate of cellular division and the levels of acid and alkaline RNase activity was again obvious in this series of rat tissues, with one exception—kidney tissue. This tissue, which has a very low rate of cellular division, had the highest level of acid RNase and the second highest for the alkaline enzyme. If the ratio of alkaline to acid RNase was considered, kidney tissue was no longer an exception. A very high ratio was obtained for intestinal mucosa, and decreasing values were obtained for the other tissues classified according to their rate of cellular division.

From these data it would seem that a correlation may exist between the levels of acid and alkaline RNase in a tissue, or at least between their ratios of activity and their respective rates of cellular division. However, more results on other tissues are needed on this question.

As noted above, the activity of alkaline RNase was always greater than the activity of the acid enzyme (TABLES 1 and 2). In fact, the lowest ratio of alkaline to acid RNase observed was 2.2. This is not in accord with the results of Roth[1] and Maver and Greco,[5] who obtained greater activity at acid *p*H than at alkaline *p*H. These apparent discrepancies can be explained by the fact that different methods of assay were used in determining RNases activity. Maver and Greco used, as precipitating agent, perchloric acid at a final concentration of 3 per cent, while we used a 2 per cent concentration. The nature of the substrate was another important point of difference. Maver and Greco employed RNA prepared by the method of Grinnan and Mosher,[9] which yields a highly polymerized substance; commercial preparations of RNA were employed in our determinations. In the method of Roth, ethanol-HCl was used as precipitating agent.[1] Under these conditions, it is most probable that only the mononucleotides remained in solution. Some assays carried out under our conditions, but with trichloracetic acid-uranyl acetate as precipitating agent, showed that the activity at acid *p*H was greater than at alkaline *p*H. These facts suggested that the reaction products of acid and alkaline RNases were different, and lead us to study the action of these enzymes on RNA. These results will be discussed in the last part of this paper.

Intracellular distribution. The intracellular distribution of acid and alkaline RNase has been studied in cellular fractions isolated by differential centrifuga-

tion from sucrose homogenates of the following tissues: intestinal mucosa, kidney, and liver. The percentage distributions in the various cellular fractions are given in TABLE 3. The percentage distributions of both the acid and alkaline RNases were the same in a given tissue. However, this distribution varied from one tissue to another. Acid RNase was found chiefly in the soluble fraction of intestinal mucosa and kidney, in percentages of 43.1 and 44.3, respectively. Similar results have been obtained by Reid and Stevens.[10] The mitochondrial fraction of liver undoubtedly contained the bulk of the enzymatic activity. The same results were obtained for alkaline RNase.

The intracellular distribution of liver RNase determined at acid *p*H is similar to that reported by Schneider and Hogeboom[4] who, however, determined the activity at *p*H 5.0 instead of 5.5. These authors observed a very low activity

TABLE 3

PERCENTAGE DISTRIBUTION OF RNASES IN CELLULAR FRACTIONS ISOLATED FROM RAT TISSUE HOMOGENATES

Tissue	Cell fractions*					
	H	N	M	Mc	S	Percentage recovery
Acid RNase						
Intestinal mucosa	100	3.1	18.1	22.9	43.1	87.2
Kidney cortex	100	24.5	22.1	8.5	44.3	99.4
Liver	100	11.3	54.2	15.5	13.1	94.1
Alkaline ribonuclease						
Intestinal mucosa	100	5.7	16.3	17.2	44.2	83.4
Kidney cortex	100	21.8	30.5	14.9	33.8	101.0
Liver	100	13.8	46.8	18.6	16.7	95.9

* The cellular fractions, namely nuclear (N), mitochondrial (M), microsomal (Mc) and supernatant fluid (S), were isolated from tissue homogenates (H) by differential centrifugation.[10] The activities are the mean values for two to six animals.

in the nuclear fraction and concluded that the nucleus of the liver cell was devoid of any activity, taking into account the proportion of the number of mitochondria present in this fraction. They also carried out determinations of activity on purified nuclear preparations,[4] and found that the activity in the nuclei was less than 1 per cent of the homogenate activity. Similar studies, in our hands, revealed that the activity of the nuclear fraction, corrected for the number of mitochondria present is still 5.9 and 9.1 per cent of the total homogenate activity. Furthermore, assays carried out on purified nuclear preparations showed that these preparations contained 8.0 and 9.6 per cent of the total homogenate activity. In view of this, it is difficult to rule out the presence of RNases in the liver cell nucleus, but it would seem that the nuclear activity is negligible. In kidney, however, the remaining activity of the nuclear fraction after correction for contaminating mitochondria (20 per cent of the total number) cannot be disregarded. In fact, this corrected activity corresponds to 20.1 and 15.7 per cent of the total homogenate activity. It

would thus seem that the question of RNase activity in the cell nucleus is not yet resolved and that some tissues may contain nuclear activity whereas others may not. A protein with nuclease activity toward the synthetic adenosine monophosphate polymer has been isolated from guinea pig liver nuclei.[11]

The presence of RNase in mitochondria has been questioned by de Duve *et al.*[12] These authors claimed that many hydrolytic enzymes, including acid RNase, were present in the lysosomes, a class of cell particulates intermediate

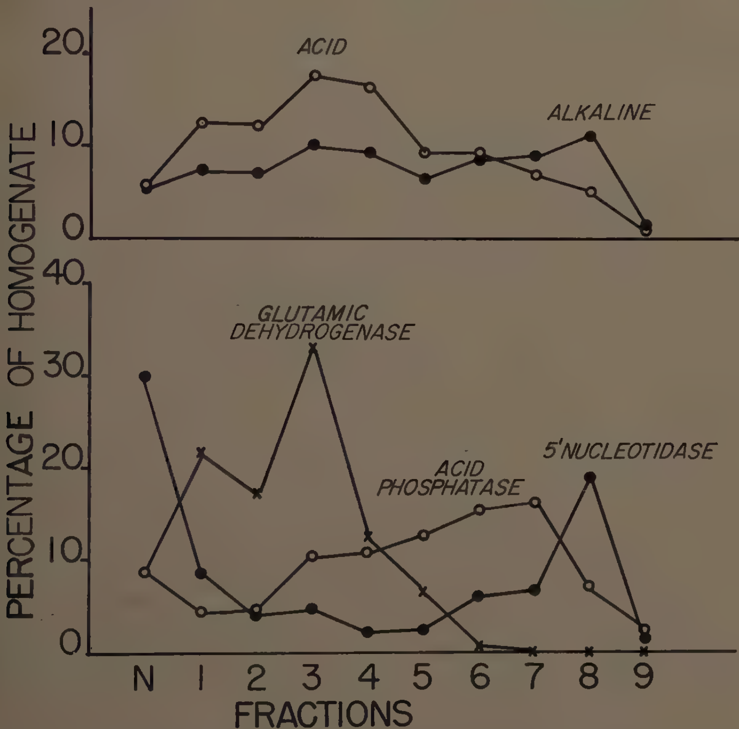

FIGURE 3. Percentage distribution of acid and alkaline RNases as compared to glutamic dehydrogenase, acid phosphatase, and 5′ nucleotidase in cellular fractions isolated from homogenates prepared in 0.88 M sucrose.

in size between mitochondria and microsomes. A detailed study of the distribution of RNases in numerous cellular fractions has been carried out in our laboratory using a modified procedure of Novikoff,[13] for differential centrifugation. The results (FIGURE 3) show that acid RNase was concentrated in Fractions 3 and 4, which correspond to mitochondria as ascertained by electron microscopy of these fractions and by the high concentration of glutamic dehydrogenase, a mitochondrial enzyme. Alkaline RNase was more evenly distributed and was also found in Fraction 8 (corresponding to the microsomes) as shown by the high concentration of 5′ nucleotidase in this fraction. The distribution curves of RNase activities were different from the curve of acid phosphatase, which was present in the lysosomes (Fractions 6 and 7), as claimed

by de Duve. From our results it is concluded that acid RNase of liver is concentrated in the mitochondrial fraction.

Conclusions from the studies on normal tissues. The data presented in this first part lead to the observation that a possible relation may exist between the ratio of alkaline to acid RNase activity of a tissue and its rate of cellular division. The data presented on the intracellular distribution indicated that the nuclear fraction of the cell may contain RNase activities—but in varying proportions from tissue to tissue. The mitochondrial fractions were shown to contain a large proportion of the activities of acid and alkaline RNases, especially in liver.

Studies on Liver Ribonucleases

Azo-dye feeding, tumors, and regeneration. Previous studies carried out in our laboratories on liver RNase and desoxyribonuclease (DNase) during feeding of an azo dye and during regeneration following partial hepatectomy have shown that the data on the activities of these enzymes apparently were influenced by expressing the results on a nitrogen basis. However, when the activities were calculated per average cell, no change was apparent. The variations previously observed were due apparently to a change in the density of the cell population of the liver. This fact, the findings that 2 RNases were present in rat liver,[1, 2] and the possibility that their mode of action on RNA was different[2] prompted us to reinvestigate the behavior of these enzymes in the same conditions.

Since the density of the cell population in the liver is an important consideration in assessing the variations of a liver enzyme under various conditions, the results were expressed per average nucleus. Another factor that should not be overlooked is the variations in the proportions of the various cell types in liver.[14] In the case of RNase of liver, Wattieaux *et al.*[15] have shown that this enzyme was present chiefly in the parenchymal cells.

TABLE 4 shows the levels of acid and alkaline RNases in the liver of rats fed 4-dimethylaminoazobenzene (DAB), in primary liver tumor, and in the Novikoff hepatoma. The level of acid RNase activity per average nucleus is not affected in the DAB-fed animals as compared to the basal-fed ones. The basal diet, which is low in protein, slightly decreased the enzymatic activity in the 80-day group. These results are in accordance with those of Schneider *et al.*,[16] who employed the same diet and the carcinogen 3′-methyl-4-dimethylaminoazobenzene.

Roth observed no variation in the specific activity of acid RNase of mitochondrial fractions isolated from livers of rats fed acetylaminofluorene for as long as 16 weeks, whereas the alkaline enzyme decreased by 50 per cent in the same animals.[17] Our results showed an increased specific activity of the acid RNase of the mitochondrial fractions isolated from the liver of animals fed DAB, whereas the alkaline RNase activity remained constant.[8] This is in accordance with the results of Schneider *et al.*[16] who reported an increased specific activity of the acid RNase of the mitochondrial fractions.

The acid and alkaline RNase activities in primary liver tumors were slightly higher than in rat liver. On the other hand, these enzyme activities were much

lower in the Novikoff hepatoma; the hepatoma cell contained merely 20 per cent of the activity in liver. This suggests that the catabolism of RNA may be decreased in the Novikoff hepatoma, as was observed for the catabolism of purine and purine derivatives in the same tumor.[18-20]

Greenstein previously reported a normal specific activity for acid RNase in transplanted hepatoma.[21] Similar results have been obtained by us,[8] contrary to the report of Maver and Greco,[5] who observed an increase in the specific activities of acid and alkaline RNases in 2 types of transplanted hepatoma. In our study, an increase in the specific activity of the alkaline RNase was observed in 2 of 6 Novikoff hepatomas. Nevertheless, on a per nucleus basis, the activities reported by Maver and Greco would most probably be lower than in liver.

TABLE 4

LEVELS AND PERCENTAGE DISTRIBUTIONS OF ACID AND ALKALINE RNASES IN THE LIVER OF RATS FED DAB, IN DAB-INDUCED TUMOR AND IN NOVIKOFF HEPATOMA

Tissues*	Acid RNase Homogenate activity per average nucleus × 10⁶	Percentage distribution of both acid and alkaline RNases*				Alkaline RNase Homogenate activity per average nucleus × 10⁶
		Cell fractions†				
		N	M	Mc	S	
Liver (5)	2.2 ± 0.4	13 ± 3	49 ± 7	17 ± 3	18 ± 3	4.4 ± 1.2
40 days basal-fed (3)	2.0 ± 0.4	*Recovery:* 97 ± 5				3.9 ± 1.0
40 days DAB-fed (5)	2.6 ± 0.4					3.8 ± 1.4
80 days basal-fed (4)	1.5 ± 0.4					3.3 ± 1.0
80 days DAB-fed (4)	1.7 ± 0.4					2.2 ± 0.8
Primary tumor (5)	3.1 ± 1.5	13 ± 2	31 ± 5	9 ± 1	30 ± 4	5.3 ± 2.1
Novikoff hepatoma (A) (6)	0.5 ± 0.2	*Recovery:* 83 ± 5				0.8 ± 0.2
(B) (2)						3.7 ± 1.4

* Experimental conditions and percentage distributions of acid and alkaline RNases were as reported in detail in Allard *et al.*[8]

† Key: N, nuclear; M, mitochondrial; Mc, microsomal; and S, supernatant fluid.

The percentage distribution of acid and alkaline RNases in the various cellular fractions isolated from the tissues mentioned above is shown in TABLE 4. The intracellular distribution was the same for both enzymes in normal liver and in liver of basal- or DAB-fed animals.[8] The percentage distributions computed from all the results obtained (shown in the center of TABLE 4) thus apply for acid as well as alkaline RNases. The mitochondrial fraction always contained the largest proportion of RNase activity.

In the primary tumor and in the Novikoff hepatoma, the distribution pattern differed from the normal. The acid and alkaline RNase activities of the mitochondrial and microsomal fractions were about one half those observed in the corresponding cellular fractions isolated from normal livers. This loss was somewhat compensated by increased activity in the soluble fraction of the cell. The lower recoveries obtained with the tumor tissue as compared to liver may be explained by an increased concentration of inhibitor in the tumors, as

could be implied from the results of Roth, who reported a greatly increased concentration of inhibitor in the livers of rats fed AAF for 16 and 20 weeks.[17]

From the results presented it would seem that the activity of acid and alkaline RNases per average nucleus is slightly affected by the feeding of a low protein diet, but not by the azo dye. These enzymatic activities are greatly decreased in the transplanted tumor. The intracellular distribution of these enzymes in primary liver tumor and in Novikoff hepatoma was different from that for normal liver.

Recently, Reid and Lotz[22] reported on the behavior and intracellular distribution of RNases in the liver of rats fed azo dye and in primary liver tumor. Their results on acid RNase are in reasonable agreement with ours, whereas the data on alkaline RNase are mostly different. This might be explained by the fact that Reid and Lotz destroyed the tissue RNase inhibitor.

TABLE 5
LEVELS AND PERCENTAGE DISTRIBUTIONS OF ACID AND ALKALINE RNASES IN REGENERATING RAT LIVER

Days after operation*	Acid RNase Homogenate activity per average nucleus $\times 10^6$	Percentage distribution of both acid and alkaline RNases* — Cell fractions†				Alkaline RNase Homogenate activity per average nucleus $\times 10^6$
		N	M	Mc	S	
0.75 laparotomy	2.0 ± 0.1	17 ± 2	49 ± 7	18 ± 4	13 ± 2	3.2 ± 0.3
0.75 hepatectomy	2.2 ± 0.6	*Recovery:* 97 ± 8				3.8 ± 0.9
1 laparotomy	1.2 ± 0.4					2.7 ± 0.8
1 hepatectomy	1.4 ± 0.1					3.3 ± 0.4
3 laparotomy	1.8 ± 0.1					3.7 ± 0.1
3 hepatectomy	1.9 ± 0.2					3.7 ± 0.5
8 laparotomy	1.5 ± 0.2					2.7 ± 0.1
8 hepatectomy	1.8 ± 0.3					3.5 ± 1.0

* The experimental conditions and percentage distributions of acid and alkaline RNases in each tissue were as reported in Allard *et al.*[8]
† Key: N, nuclear; M, mitochondrial; Mc, microsomal; and S, supernatant fluid.

Regenerating liver. The study of regenerating liver has been undertaken to find if a high rate of cellular division could influence the activity and intracellular distribution of acid and alkaline RNases. Laparotomized rats were used as controls for the hepatectomized animals. TABLE 5 shows that the levels of activity of both enzymes in the liver of hepatectomized rats were similar to those of the controls.

The percentage distribution of the enzymes in the various cellular fractions was determined for each group of animals at each period.[8] No variations were observed for either enzyme. Consequently, a mean distribution has been computed from these values (TABLE 5). The distribution pattern shows that the greater proportion of enzymatic activity was found in the mitochondrial fraction; the distribution was similar to that observed in normal liver. Thus, a high rate of cellular division in liver apparently does not influence the levels or the intracellular distribution of acid and alkaline RNases. Regenerating liver would be an exception to the observation that a possible relation existed

between the rate of cellular division in a normal tissue and the ratio of alkaline to acid RNase activity.

Fasting. The study of the effect of fasting on acid and alkaline RNases of liver was initiated as an extension of studies conducted on hepatoma cells.[8] It was thought that this study might help in understanding the biochemical behavior of hepatoma cells. TABLE 6 shows that the activities of acid and alkaline RNases of liver were slightly lower after 1 and 7 days of fasting as compared to the levels observed in the liver of fed rats. The loss of activity was about 20 per cent per average nucleus. This is of interest in view of the fact that fasting induced a loss of cytoplasm and of 50 per cent of the activities of such enzymes as uricase, inosine phosphorylase, adenosine triphosphatase, and cathepsin. This has been discussed fully in a recent publication from our laboratory.[24]

TABLE 6

LEVELS AND PERCENTAGE DISTRIBUTIONS OF ACID AND ALKALINE RNASE IN THE LIVER OF FED AND FASTED RATS

Tissues*	Acid RNase Homogenate activity per average nucleus × 10^6	Percentage distribution of both acid and alkaline RNases† — Cell fractions‡ N	M	Mc	S	Alkaline RNase Homogenate activity* per average nucleus × 10^6
Fed	1.5 ± 0.3	16 ± 2	47 ± 4	16 ± 4	13 ± 3	2.4 ± 0.6
Fasted 24 hours	1.2 ± 0.2	*Recovery:* 93 ± 6				1.9 ± 0.3
Fed	1.7 ± 0.3					3.3 ± 1.0
Fasted 7 days	1.3 ± 0.3					2.8 ± 0.1

* The experimental conditions were as described in de Lamirande *et al.*[20] and Greenstein.[21]

† The percentage distributions of acid and alkaline RNases in each tissue were as reported in detail in de Lamirande *et al.*[20]

‡ Key: N, nuclear; M, mitochondrial; Mc. microsomal; and S, supernatant fluid.

The intracellular distributions of acid and alkaline RNases were similar whether the animals were fed or fasted.[25] A mean of these intracellular distributions was computed and is shown in TABLE 6. The greatest activity was found in the mitochondrial fraction as observed for normal liver. The results obtained show that fasting slightly lowers the activities of acid and alkaline RNases per average liver cell, but does not affect their intracellular distributions.

Effect of cortisone. It has been shown that cortisone administration disturbed the distribution of RNA in the liver cell, and there was some suggestion that the structure of RNA also might have been affected.[26] This prompted us to study the effect of cortisone injections on the levels and intracellular distributions of acid and alkaline RNases in liver. Control animals were injected with the "vehicle" employed to suspend the cortisone. TABLE 7 shows that the levels of acid and alkaline RNases per average liver nucleus were not affected by cortisone administration as compared to controls—whether the animals were fed or fasted overnight. The slight decrease in liver acid RNase activity

and the increase in alkaline RNase activity observed in the cortisone group that was fasted overnight were not statistically significant.

The intracellular distributions of acid and alkaline RNases were determined for each group of animals. A slight change was observed in the percentage activity of the microsomal fraction isolated by differential centrifugation from the liver of fed rats treated with cortisone. However, this change was not manifest in rats fasted overnight. It would thus seem that cortisone administration was without significant effect on the distribution of RNases.

Conclusions on liver studies. The data, briefly summarized here, on the activity of RNases of the livers of rats under various physiological and pathological conditions illustrate the stability of these intracellular enzymes. The apparent variations in activity when expressed per wet weight or per milligram of nitrogen were only a reflection of the variations of the cell density or of the protein content of the liver.

TABLE 7

EFFECT OF CORTISONE ADMINISTRATION ON THE LEVELS AND PERCENTAGE DISTRIBUTIONS OF ACID AND ALKALINE RNASE ACTIVITIES OF RAT LIVER

Material injected*	Acid RNase Homogenate activity per average nucleus $\times 10^6$	Percentage distribution of both acid and alkaline RNases — Cell fractions†				Alkaline RNase Homogenate activity per average nucleus $\times 10^4$
		N	M	Mc	S	
Vehicle fed	1.6 ± 0.4	16 ± 2	46 ± 5	21 ± 7	12 ± 2	2.2 ± 0.1
Cortisone fed	1.3 ± 0.2	16 ± 2	49 ± 6	11 ± 3	12 ± 2	2.2 ± 0.1
Vehicle fasted	1.9 ± 0.4	18 ± 2	37 ± 6	20 ± 3	14 ± 3	3.1 ± 1.0
Cortisone fasted	1.4 ± 0.3	18 ± 2	42 ± 8	17 ± 3	14 ± 3	4.6 ± 2.1

* The experimental conditions and percentage distributions of acid and alkaline RNases in each tissue were as reported in detail in Allard *et al.*[23]

† Key: N, nuclear; M, mitochondrial; Mc, microsomal; and S, supernatant fluid.

The stability of acid and alkaline RNases is illustrated by the fact that in such a drastic condition as a 7-day fast the activities of these enzymes decreased by only 20 per cent. This behavior is comparable to only a few other enzymes among those investigated in fasted animals.[24] The stability of these enzymes is further emphasized by their unaffected activities and intracellular distributions in regenerating rat liver and in the liver of rats injected daily with massive doses of cortisone.

The only important variations in the levels of activity as well as in the intracellular distributions of the acid and alkaline RNases were observed in liver tumors. In these tissues the mitochondrial and microsomal fractions had low activities, but the soluble fraction of the cell contained a greater activity. This can be correlated with the low amount of organized material in these tumor cells as illustrated by their low content of nitrogen and mitochondria[8] and of phospholipids.[27, 28]

These studies, carried out to gain information on the physiological role of RNases, did not produce spectacular results. The only conclusion that can be

formulated is that the levels and intracellular distributions of RNases are not easily influenced—at least, in liver.

Properties of Isolated RNases of Liver

Methods for the separation and partial purification of acid and alkaline RNases of rat liver have been devised in our laboratory by Zytko *et al.*[29] Preparations of acid RNase were 36 times more concentrated than in rat liver homogenate, and were freed from alkaline phosphatase, adenosine triphosphatase, and unspecific diesterase. However, these preparations were slightly contaminated by alkaline RNase and traces of acid phosphatase. Preparations of alkaline RNase were concentrated 34 times as compared to rat liver

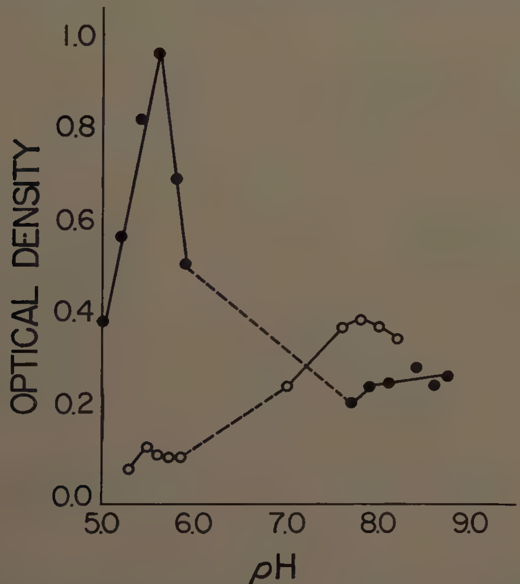

FIGURE 4. The *p*H-activity curve of partially purified preparations of acid RNase (*closed circles*) and alkaline RNase (*open circles*).

homogenate, and were freed from acid RNase, unspecific acid and alkaline phosphatase, adenosinetriphosphatase, and unspecific diesterase.

FIGURE 4 shows the *p*H-activity curve of the acid and alkaline RNase preparations. The partially purified acid RNase showed an optimal activity at *p*H 5.7, whereas partially purified alkaline RNase had an optimal *p*H between 7.5 and 8.0. These optimal *p*Hs were similar to those observed with whole liver homogenates. As in liver homogenates,[30] acid RNase was completely destroyed by heating for 5 min. in boiling water, whereas alkaline RNase apparently was not affected by such treatment. Similar results were obtained by Roth.[31] Furthermore, acid RNase was half inactivated by lyophilization of its solutions, whereas alkaline RNase was unaffected.

Some preliminary data on the chemical properties of purified preparations of acid and alkaline RNases have been obtained. The results in TABLE 8 illus-

TABLE 8

PAPER CHROMATOGRAPHY OF CYCLIC MONONUCLEOTIDES INCUBATED WITH LIVER RNASES

R_f* Values of the Various Spots

	Incubation time, hours		
	0	17	40
Adenylic acid			
Controls			
Ap!	0.11–0.12	0.11–0.12	0.11–0.12
A3p	0.18–0.19	0.18–0.19	0.18–0.19
A2p	0.28–0.31	0.28–0.31	0.28–0.31
Incubation mixture			
Ap! and acid RNase, *p*H 5.7		0.11 0.28–0.31	0.28
Ap! and heated acid RNase, *p*H 5.7		0.11	0.11
Ap! and alkaline RNase, *p*H 7.8		0.12	0.11
Uridylic acid			
Controls			
Up!	0.52–0.55	0.55	0.55
Uridylic acid†	0.68–0.70	0.68	0.70
Incubation mixtures			
Up! and acid RNase, *p*H 5.7		0.55	0.55 0.70
Up! and heated acid RNase, *p*H 5.7		0.55	0.55
Up! and alkaline RNase, *p*H 7.8		0.50	0.51

* Solvent was composed of saturated ammonium sulfate:isopropanol:water (79:2:19 V/V/V).

† Commercial uridylic acid containing both U2p and U3p. The solvent system used did not permit the separation of these isomers; only one spot was then identified on the chromatogram.

TABLE 9

PHYSICAL CHARACTERISTICS OF MONONUCLEOTIDES LIBERATED FROM RNA BY LIVER ALKALINE RNASE

	Before acid hydrolysis	After acid hydrolysis					
				Spectrophotometric data‡			
	R_f value in Solvent 1*	R_f value in Solvent 1	R_f value in Solvent 2†	λ max.	λ min.	250/260	280/260
Peak 2	0.52	0.73–0.74	0.61–0.62	271	250	0.89	0.90
Peak 3	0.52	0.73–0.74	0.79–0.82	260	239	0.80	0.30
Cp!	0.52	0.73–0.74	0.61–0.62	272	250	0.90	0.85
Up!	0.52	0.73–0.74	0.79–0.82	261	242	0.83	0.28

* Solvent 1 was composed of saturated ammonium sulfate:1 M sodium acetate:isopropanol (79:19:12).

† Solvent 2 was composed of isopropanol and 2 *N* hydrochloric acid.

‡ Determined in alkaline medium, *p*H 11.6.

trate the behavior of these enzymes in the presence of cyclic 2′-3′-mononucleotides. Acid RNase was able to split Ap! to give A2p; it was also able to split Up!. Heated acid RNase preparation was without effect on either substrate; alkaline RNase was unable to split Ap! or Up!, even after 40 hours of incubation. Similar results were obtained with Cp!. These results have been confirmed and extended by Nodes and Reid.[32]

The action of acid and alkaline RNases on rat liver RNA also was investigated.[33] The elution patterns of the reaction products were generally similar to those obtained by the action of pancreatic crystalline RNase, but there were differences. Two peaks eluted from the reaction products of the action of rat liver alkaline RNase on rat liver RNA were cyclic pyrimidine mononucleotides as shown in TABLE 9. The R_f values of these 2 peaks corresponded to those of cyclic mononucleotides. After hydrolysis with 0.1 N hydrochloric acid, their R_f values and spectrophotometric characteristics were analogous to those of the noncyclic cytidylic and uridylic acids. The fact that alkaline RNase produced only cyclic pyrimidine mononucleotides agrees with the results presented in TABLE 8, which showed that this enzyme could not split cyclic mononucleotides.

The study of the action of acid RNase on rat liver RNA showed that this enzyme liberated noncyclic purine mononucleotides as well as noncyclic pyrimidine mononucleotides. This was ascertained by rechromatography on paper of the eluted peaks obtained from ion-exchange chromatography. The analysis of the other reaction products is under way.

From the results briefly mentioned here, it would seem that the two rat liver RNases are different enzymes having their own specificity.

General Conclusions

The results summarized in this presentation indicate that the relation between the behavior of intracellular RNases and the physiology of the cell are far from understood. In the first part of this article, suggestion has been made of a possible relation between the ratios of alkaline to acid RNase activity and the rates of cellular division of normal tissues. In the second part, the great stability in the levels and intracellular distributions of both liver acid and alkaline RNases, was illustrated. Great variations in activity were evident, however, in liver tumors, but the meaning of these changes was not clear.

Results described in the third part showed that rat liver acid and alkaline RNases are most probably two different enzymes that split specific bonds of the RNA molecule. It is hoped that the knowledge of the mode of action of RNases will help to clarify their intracellular function.

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INTRACELLULAR LOCALIZATION AND CHROMATOGRAPHY OF MOUSE PANCREAS RIBONUCLEASES*

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A number of tissues have been homogenized in 0.25 M sucrose, and various intracellular particulates have been separated by differential centrifugation according to the general procedure of Schneider and Hogeboom,[1] or modifications thereof.[2] In many instances, the distribution of enzymes in these fractions has yielded interesting data on the localization of a given enzyme or substance and on its presumed metabolic function. As a result of this work, it has been demonstrated that certain cellular components are present exclusively in certain particulates. Thus, desoxyribonucleic acid (DNA) has been found only in nuclei,[3] certain enzymes of the respiratory chain (succinoxidase and cytochrome oxidase) have been found in mitochondria,[1] and glucose-6-phosphatase has been detected in liver microsomes.[4] It becomes possible, therefore, if one assumes a similar intracellular enzyme localization in other tissues, to utilize enzyme activities as "markers" for organelles. Van Lancker and Holtzer[5] have recently applied such reasoning in a study of the intracellular distribution of DNA, ribonucleic acid (RNA), amylase, desoxyribonuclease (DNase), cytochrome oxidase, and acid phosphatase in mouse pancreas.

In other work we have reported that mouse pancreas ribonuclease (RNase) can be chromatographically separated into two major fractions and one minor fraction.[6] It became possible, therefore, to determine whether each chromatographic peak was derived from one intracellular site, or whether a specific organelle contained more than one type of chromatographically distinct RNase. In the course of these studies it was observed that certain treatments exerted marked effects on the chromatographic behavior, as well as on the total RNase activity of some of the fractions. These results will be presented and their significance discussed.

Methods and Materials

Fractionation procedure. CBA mice, 2 to 6 months old, were used in this investigation. Approximately 30 were sacrificed by severing the spinal column, and the pancreases were immediately excised and freed from connective tissue. They were homogenized in 9 volumes of ice-cold 0.25 M sucrose in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The various particulate fractions were obtained by differential centrifugation in a Spinco Model L centrifuge. An outline of the centrifugal forces, the number of washings, and terminology is presented in TABLE 1.

Chromatographic procedure. Each particulate fraction was suspended in 4 ml. of 0.2 M phosphate buffer, *p*H 6.47; this was divided into 2 equal portions.

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To one portion was added sufficient 1 N H_2SO_4 to bring it to a final concentration of 0.25 N H_2SO_4. The precipitate was centrifuged, the cloudy supernatant solution adjusted to pH 5.8, and the suspension centrifuged again.[7] The clear supernatant solution was placed on a 0.9 × 30-cm. column of the cation exchange resin IRC-50 (XE-64) prepared as described by Hirs,[8] and the column then percolated with 0.2 M phosphate, pH 6.47. The other half of the suspension was centrifuged once for 10 min. at 600 g, and the supernatant solution placed directly on the column and eluted as described above. The eluates (1 ml. each) were obtained by means of an automatic fraction collector.

Quantitative procedures. The original homogenates, solutions to be chromatographed, and eluates were assayed for RNase activity at pH 5.0 or pH 7.5 by the procedure of Dickman *et al.*[9] Under the conditions of the standard assay, an absorbancy unit is defined as the value of the experimental absorbance (A_{260}) multiplied by the dilution factor. Ninhydrin-positive material was

Table 1
Fractionation Scheme for Isolation of Nuclear and Cytoplasmic Particulates from Mouse Pancreas Homogenates Prepared in 0.25 M Sucrose Solution

Cell fraction	g × time (min.)	No. of washes
Nuclear-dense granule	6,000	4
Cytoplasmic		
a principal zymogen granule	8,400	2
b mitochondria	16,800	2
c mitochondria	90,000	2
d mitochondria	180,000	1
e microsomes	411,900	1
f microsomes	1,580,000	1
g microsomes	3,150,000	1
h postmicrosomal	6,300,000	0
i final supernatant		

measured by the procedure of Yemm and Cocking.[10] Nitrogen content of isolated fractions was determined by the semimicro-Kjeldahl procedure of Hiller *et al.*[11]

Results

Chromatography of mouse pancreas extracts. Fresh mouse pancreas was homogenized in 9 volumes of cold 0.25 N H_2SO_4, and the turbid solution treated according to the procedure of Hirs *et al.*[7] The supernatant solution was placed on a column of XE-64 resin and elution was accomplished with 0.2 M phosphate (pH 6.47). Aliquots of 1-ml. fractions were analyzed for RNase activity at pH 5.0 and for ninhydrin-positive substances. The elution patterns are presented in FIGURE 1*b*. The enzyme activity was eluted as 2 major peaks, with peak volumes at 14 and 36 ml., respectively. Ninhydrin-positive substances were readily eluted as a double peak at 10- and 15-ml. elution volume. Most of the ninhydrin-reacting material was readily dialyzable, and presumably had no direct connection with the RNase activity. In addition to these 2

types of materials, a third, which possesses an absorption peak at 258 mμ, was routinely found at an elution volume of 8 to 10 ml.

The phosphate buffer extract of whole pancreas produced a very different elution pattern (FIGURE 1*a*). RNase activity was again eluted in 2 major

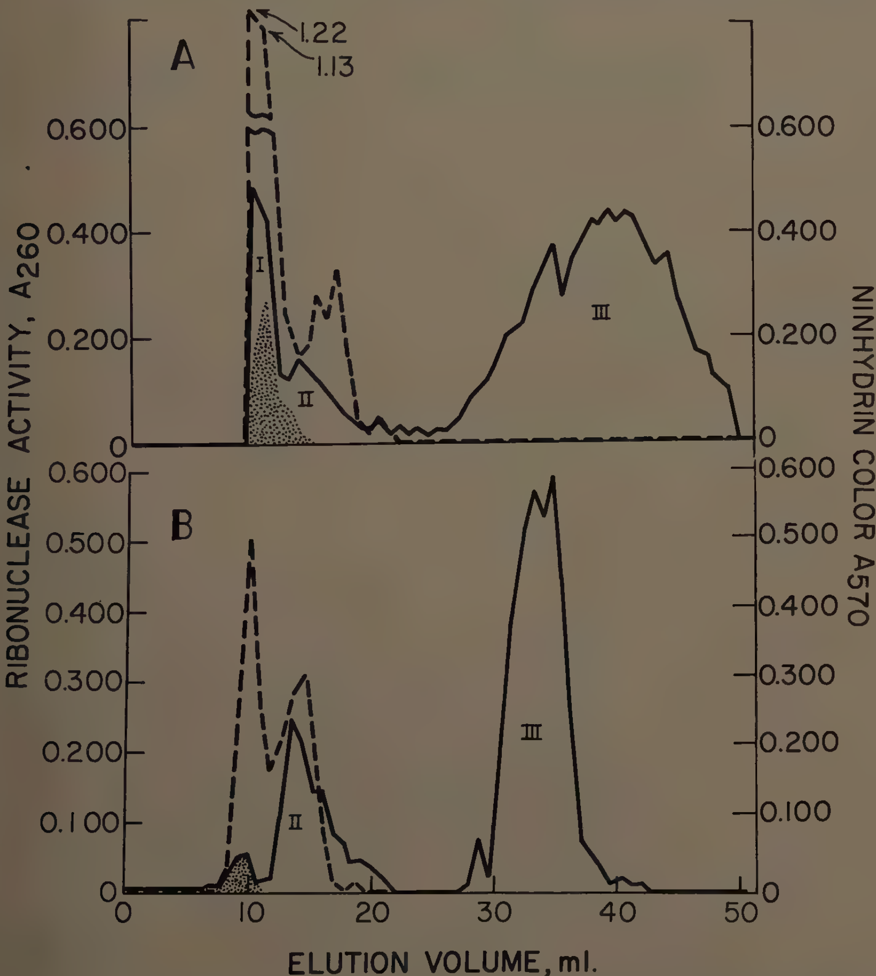

FIGURE 1. Chromatographic elution patterns of RNase activity and ninhydrin-positive substances obtained from homogenates. (*A*) Pattern obtained with phosphate buffer extract; (*B*) with H_2SO_4 treatment. Solid line is for RNase activity, *p*H 5.0; dashed line for ninhydrin color. Shaded area represents polynucleotide material.

fractions, but the shapes of the peaks and their elution volumes were altered from the acid-treated samples. A large peak of RNase activity was not retained by the column (Peak I). This peak was essentially absent in the eluates from the acid-treated solutions. In addition, Peak III was much broader in the phosphate extract than in the acid-treated sample. Ninhydrin-positive

and 258 mμ-absorbing material were both present in higher concentrations than in the eluates obtained after acid treatment of the tissue.

Ninhydrin-positive material in the region of Peak III of both samples was negligible; this is not surprising when one considers the sensitivity of the assay: 0.28 γ of RNase results in an A_{260} of 0.25 in the *p*H 5 assay. This amount of

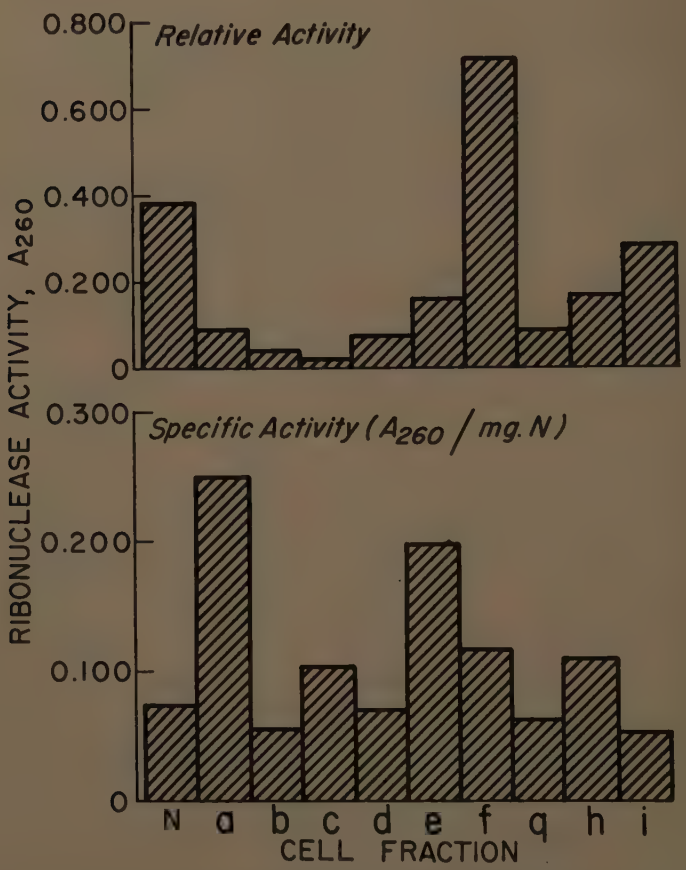

FIGURE 2. Intracellular distribution of mouse pancreas RNase activity. "Relative activity" indicates the RNase activity per fraction, as determined at *p*H 5.0. "Specific activity" is calculated as indicated. The fractions are described in TABLE 1.

enzyme, on the assumption that it possesses a molecular weight of 14,000, is equivalent to 2×10^{-5} μmoles. If there are 10 lysine residues and one terminal amino group per mole, as found in beef RNase,[12] the reacting groups are still too few in number to produce a significant ninhydrin color.

Distribution of RNase activity among intracellular fractions. Each intracellular fraction was assayed for RNase activity and total N. The RNase activity per pellet is shown in FIGURE 2 (*top*). Most of the activity was present in three fractions: *f*, microsomes; *N*, nuclear-dense zymogen granule; and *i*,

supernatant solution. The zymogen granule fraction, *a*, exhibited the highest specific activity (RNase activity per milligram of N), followed by microsomal fraction, *e* (FIGURE 2, *bottom*).

From the relatively large amylase and RNase activities in the nuclear fraction *N*, it appears that it is contaminated with zymogen granules. Consequently, this residue is referred to as the nuclear-dense zymogen granule fraction; the second residue *a*, which contained the least nitrogen of any fraction[5] and possessed considerable amylase and RNase activities, is referred to as the principal zymogen granule fraction.

TABLE 2

DISTRIBUTION OF RNASE ACTIVITY IN CELL FRACTIONS OBTAINED FROM MOUSE PANCREAS HOMOGENATES PREPARED IN 0.25 M SUCROSE*

Cell fraction†	Total nitrogen, mg.	RNase activity,‡ per cent	
		*p*H 5.0 assay	*p*H 7.5 assay
Nuclear	4.0	14.5	16.7
a	0.5	11.1	6.2
b	0.9	3.0	2.7
c	0.5	1.9	1.8
d	1.0	4.2	3.6
e	1.3	10.1	5.8
f	5.8	34.6	35.5
g	2.3	5.5	4.6
h	2.0	3.5	8.2
Supernatant	9	11.5	14.1
Recovery§	94%	70%	67%
Reconstituted homogenate		96%	

* Values reported in this table represent composite data from 4 fractionation studies. Individual pellets were taken up in 1.5 ml. of 0.2 M sodium phosphate buffer, *p*H 6.47, and aliquots diluted in the appropriate assay buffer.

† See TABLE 1 for nomenclature.

‡ Determined by the standard *p*H 5.0 and 7.5 assays.

§ Recovery calculated from summation of the individual fractions as percentages of the total activity in the original homogenate.

Mitochondria, as characterized by cytochrome oxidase activity, may be considered to represent the major organelle of fractions *b*, *c*, and *d*.[5] In contrast to liver mitochondria, pancreatic mitochondrial RNase activities were very low, and the percentages of the total RNase activity exhibited by these fractions are at a minimum. A similar intracellular distribution of RNase activity in guinea pig pancreas has been reported by Siekevitz and Palade.[13]

Particle-bound RNase activity attains its maximum in the microsomal fractions. Approximately 35 per cent of the total activity is found in Fraction *f*. It is interesting to note that this fraction also contains a high amount of RNA.[5] The third major site of mouse pancreas RNase activity is the supernatant solution. This fraction exhibits 12 to 15 per cent of the activity of the total homogenate and, in addition, contains some latent RNase, as described later.

The summation of RNase activity of the individual fractions amounts to a recovery of approximately 70 per cent of the activity of the whole homogenate (TABLE 2). When the fractions were mixed to reconstitute their original

proportions in the homogenate and this solution was assayed, RNase activity equal to that of the nonfractionated homogenate was obtained.

Effect of chemical agents on RNase activity of intracellular fractions. The pellets from the washed fractions, which contained the major RNase activity, and also the supernatant solution, were diluted in 0.2 M phosphate buffer. An aliquot of each was brought to 0.25 N H_2SO_4 and processed according to

TABLE 3

EFFECT OF 0.25 N SULFURIC ACID ON RNASE ACTIVITY IN MOUSE PANCREAS EXTRACTS AND ISOLATED CELL FRACTIONS*

Cell fraction	RNase activity (pH 5) Phosphate Extract		RNase recovery, percentages
	Phosphate Extract	Acid extract	
	Absorbancy units	Absorbancy units	
Homogenate	54	52	97
Nuclear-dense granule	25.6	23.0	90
Principal zymogen granule	23.8	27.4	115
Cytoplasmic f, microsomal	43.0	55.6	132
Final supernatant	5.0	22.8	455

* The pellets from the isolated cell fractions were suspended in 1.5 ml. of 0.2 M phosphate buffer, pH 6.47, and a 0.5-ml. aliquot treated by the procedure of Hirs *et al.*[7] A 1.0-ml. portion of the pancreas extract (homogenate) or final supernatant solution was added to an equal volume of 0.2 M phosphate buffer; 0.5 ml. was then removed and treated as above. Results are averages of duplicate experiments.

TABLE 4

SOLUBILIZATION OF MICROSOMAL RNASE*

Treatment	RNase activity†			Solubilization, per cent	Recovery, per cent
	Residue	Supernatant	Total		
Sucrose, 0.25 M, pH 9	6.9	0	6.9	0	100
EDTA, 0.1 M, pH 9	0	12.4	12.4	100	185
EDTA, 0.01 M, pH 9	0	8.3	8.3	100	122
EDTA, 0.001 M, pH 9	3.2	1.6	4.8	33	70
EDTA, 0.1 M, pH 7	0.7	14.6	15.3	96	225
EDTA, 0.001 M, pH 7	4.4	1.8	6.2	29	91
NH_4Cl, 0.1 M, pH 9	0	9.8	9.8	100	144
NH_4Cl, 0.001 M, pH 9	6.2	0	6.2	0	91
NH_4Cl, 0.1 M, pH 7	4.3	2.0	6.3	32	94
Citrate, 0.1 M, pH 9	0	14.0	14.0	100	207
Urea, 6 M, pH 9	1.6	6.5	8.1	80	118

* Reproduced by permission of *Biochimica et Biophysica Acta*, New York, N. Y. Washed microsomes from a 10 per cent mouse pancreas homogenate in 0.25 M sucrose were suspended in the indicated solutions. The sediment was recentrifuged at 100,000 g for 60 min., the supernatant solutions were adjusted to pH 5.0, and both pellets and supernatants assayed for RNase activity at pH 5.0.

† Reported as absorbancy at 260 mμ per 12 ml. of microsomal suspension. Results are averages of duplicate experiments.

the method of Hirs *et al.*[7] The RNase activities of the final supernatant solutions (TABLE 3) were determined at *p*H 5.0. The activity found in the phosphate extract is taken as the standard of comparison. It can be seen that recovery of RNase activity in the whole homogenate was essentially quantitative.

The microsomal-bound RNase can be solubilized by mild procedures.[14] As shown in TABLE 4, treatment with 0.1 M ethylenediaminetetraacetic acid

TABLE 5

ACTIVATION OF SOLUBLE RNASE OF MOUSE PANCREAS*

Treatment	RNase activity†	
	A_{260} per ml.	Relative‡ per cent
Untreated	0.30	100
*p*H 5	0.23	77 (44–104)
*p*H 9 → *p*H 5	0.31	103 (77–233)
0.1 M NaCl, *p*H 9 → *p*H 5	0.85	283 (106–337)
0.1 M EDTA, *p*H 9 → *p*H 5	1.15	383 (149–567)

* Reproduced by permission of *Biochimica et Biophysica Acta*, New York, N. Y. Fresh mouse pancreas was homogenized in 0.25 M sucrose and, after removal of large particles, centrifuged for 1 hour at 100,000 g. The supernatant solution was treated as indicated. Solid NaCl or EDTA was added to bring the solution to 0.1 M, the solution was adjusted to *p*H 9 and, after 30 min. at room temperature, was adjusted to *p*H 5 and centrifuged. All assays were carried out on the clear supernatant solutions at *p*H 5.

† Reported as absorbance at 260 mμ/ml. of original supernatant solution.

‡ Figures in parentheses indicate range of relative activities in three additional experiments.

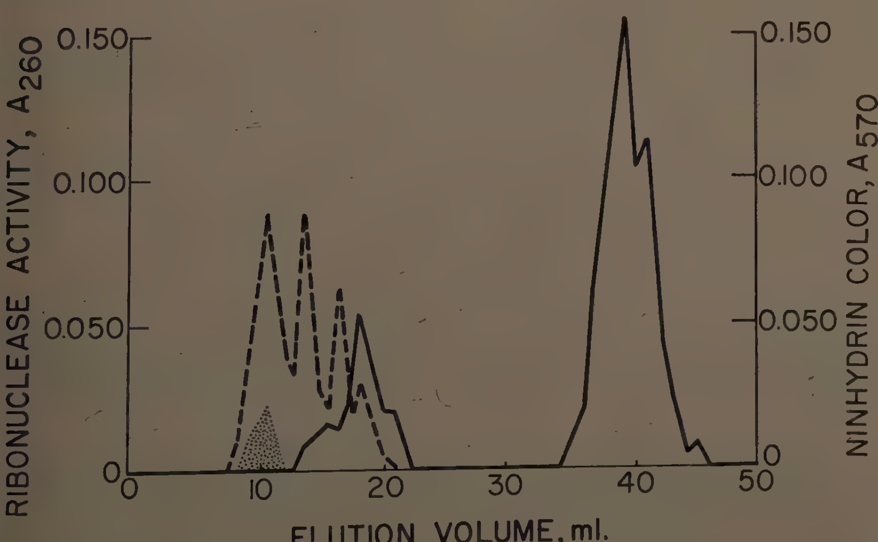

FIGURE 3. Chromatographic elution patterns of RNase activity and ninhydrin-positive substances obtained from phosphate extract of nuclear-dense zymogen granule fraction. Phosphate buffer extract. *Solid line*, RNase activity; *dashed line*, ninhydrin color.

(EDTA) at either pH 7 or pH 9 completely releases the enzyme activity from the pellet. The enzyme can also be solubilized at pH 9 by 0.1 M citrate or 0.1 M NH_4Cl; 6 M urea, or 0.001 M EDTA at pH 9, or 0.1 M NH_4Cl at pH 7 are much less effective in this regard. Elson[15] has recently reported that

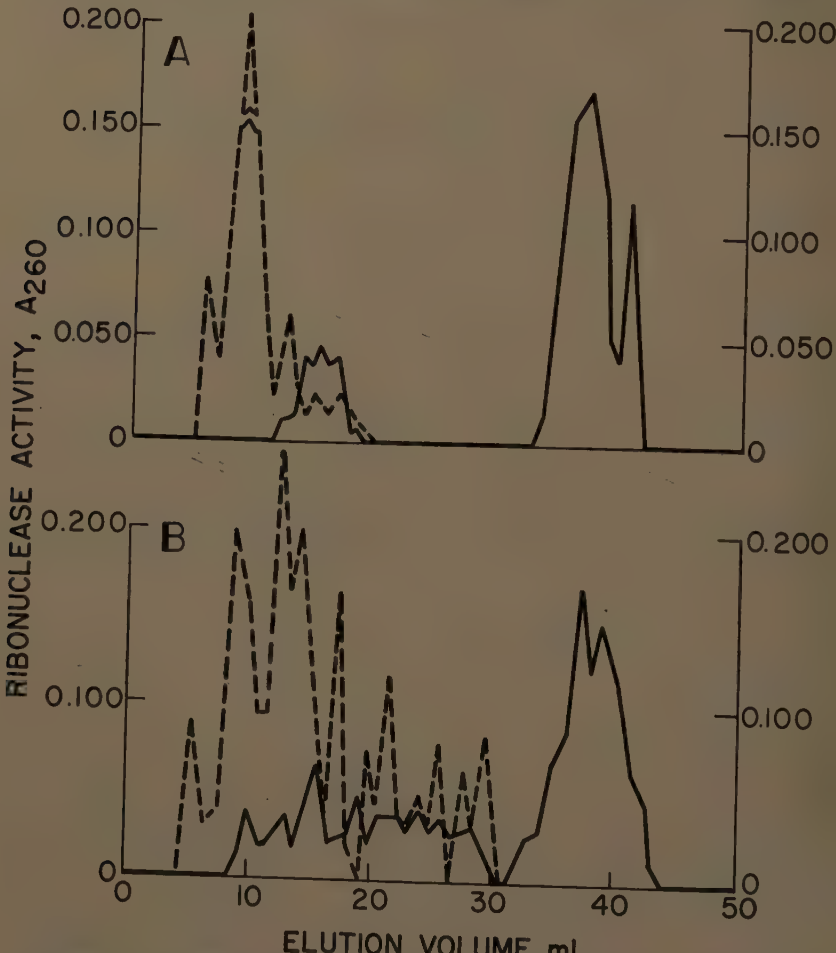

FIGURE 4. Chromatographic elution patterns of RNase activity and ninhydrin-positive substances obtained from principal zymogen granule fraction. (*A*) Phosphate buffer extract; (*B*) H_2SO_4. *Solid line*, RNase activity; *dashed line*, ninhydrin color.

treatment of ribonucleoprotein particles from *E. coli* with strong urea reveals a latent RNase. While treatment of mouse pancreas microsomes with this substance did result in a somewhat greater total activity, treatment with 0.1 M EDTA, or 0.1 M citrate resulted in a doubling of RNase activity.

Other agents, in addition to H_2SO_4, activate RNase activity in the supernatant solution.[14] As shown in TABLE 5, adjustment of the solution to pH 9, followed by acidification to pH 5 in the presence of 0.1 M NaCl or 0.1 M EDTA,

results in an average increase in the RNase activity of the solution to 3 or 4 times that of the control. When these solutions were assayed at pH 7.5, however, no increase in RNase activity was observed.

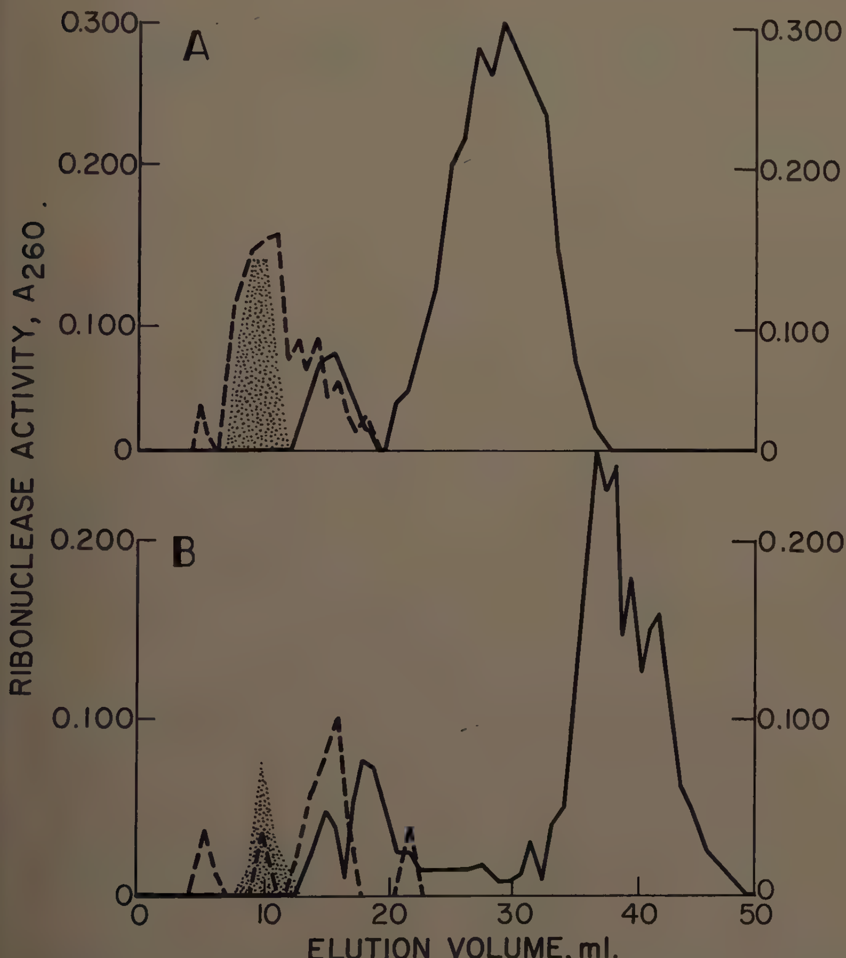

FIGURE 5. Chromatographic elution patterns of RNase activity and ninhydrin-positive substances obtained from microsomal fraction *f*. (*A*) Phosphate buffer extract; (*B*) H_2SO_4-treated. *Solid line*, RNase activity; *dashed line*, ninhydrin color; and *shaded area*, polynucleotide material.

RNase chromatographic elution patterns of pancreatic intracellular fractions. FIGURES 3, 4, 5, and 6 present the elution patterns of RNase activity and ninhydrin-positive substances from the 4 major RNase-containing fractions. The upper chart of each figure contains the elution pattern from a phosphate extract, and the lower represents that obtained after the H_2SO_4 treatment. Since the acid treatment produced no appreciable change in the elution pattern from the nuclear-dense zymogen granule fraction (FIGURE 3), it has not

been included. In the principal zymogen granule fraction (FIGURE 4), acid treatment has broadened Peak II, but had little effect on Peak III. The elution pattern from the microsome pellet, however, (FIGURE 5), exhibits a displacement or removal of the forepart of Peak III (due to acid treatment), as well as a decrease in ninhydrin-positive material and polynucleotides.

The elution patterns from the untreated and acid-treated supernatant solution are presented in FIGURE 6. The height and sharpness of Peak I in the

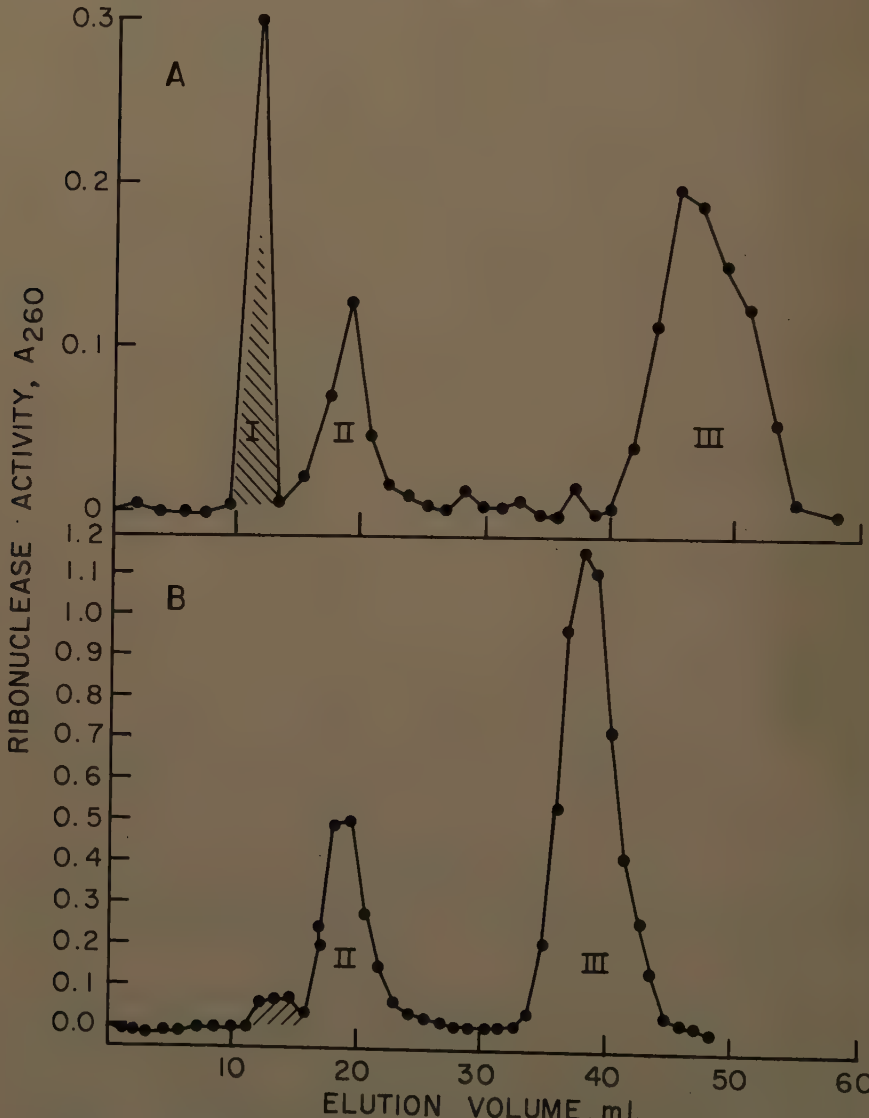

FIGURE 6. Chromatographic elution patterns of RNase activity obtained from supernatant solution. (*A*) Phosphate buffer extract; (*B*) H_2SO_4-treated. *Solid line*, RNase activity; *shaded area*, polynucleotide material.

phosphate extract are striking. It is this material, which is hardly retained by the column, that is so altered by acid treatment that it was absent from the lower elution pattern. Ninhydrin analyses were not carried out on these samples.

TABLE 6
RNASE COMPONENTS ISOLATED BY COLUMN CHROMATOGRAPHY FROM PANCREAS HOMOGENATES AND CELL FRACTIONS*

Cell fraction	RNase activity† eluted from column, per cent					
	PO_4 extract‡			H_2SO_4 extract§		
	Peak			Peak		
	I	II	III	I	II	III
Homogenate	11	10	79	3	27	70
Nuclear-dense granule	0	21	79	0	20	80
Principal zymogen granule	0	21	79	0	41	59
Cytoplasmic *f*, microsomal	0	10	90	0	21	79
Final supernatant	22	19	59	0	25	75

* Mouse pancreas was homogenized in 0.25 M sucrose, and intracellular fractions were isolated as described in the text.

† Calculated from the summation of the individual eluates of a given peak as a percentage of the total activity eluted from the column, activity was determined at *p*H 5.0. Data are average values for duplicate studies.

‡ Procedure using 0.2 M sodium phosphate buffer, *p*H 6.47.

§ Hirs *et al.*[7] procedure.

TABLE 7
RECOVERY OF RNASE ACTIVITY IN THE COLUMN EFFLUENT FRACTIONS FROM TOTAL HOMOGENATES AND CELL FRACTIONS

Cell fraction	Recovery of RNase activity,* per cent	
	Acid extract	Phosphate extract
Homogenate	67	102
Nuclear-dense granule	85	106
Principal zymogen granule	145	99
Cytoplasmic *f*, microsomal	96	143
Final supernatant	100	110

* Calculated from summation of individual eluates as a percentage of the total activity placed on the column. The data represent average values for duplicate studies; RNase activity was determined at *p*H 5.0.

TABLE 6 lists the proportions of the 3 RNase activity peaks in the various intracellular fractions suspended in phosphate buffer, and the effect of the acid treatment on them. The bulk of the activity in the homogenate, as well as that in the separated fractions, was found in Peak III. Peak I was present almost exclusively in the supernatant solution, whereas Peak II occurred in small amounts in most of the fractions. Treatment of the supernatant solution with H_2SO_4, before chromatography, resulted in the complete disap-

pearance of RNase activity in the position of Peak I. Treatment with H_2SO_4 of the particulates increased Peak II activity and diminished Peak III.

Recovery of RNase activity in chromatographic effluents. The homogenates, intracellular fractions, and the supernatant solutions—from either the H_2SO_4 treatment or the phosphate buffer extract—were chromatographed over XE-64 resin, and RNase activity was eluted with 0.2 M phosphate (*p*H 6.47). Sixty fractions (1 ml.) were collected from each preparation. A summation of RNase activity eluted from the columns is presented in TABLE 7. Recovery from the homogenate and nuclear-dense zymogen granule fraction was quantitative when these were prepared in phosphate buffer, but was low when they were subjected to the H_2SO_4 treatment.

Discussion

The results obtained in this work raise a number of interesting problems. It is obvious that H_2SO_4 treatment of either the whole tissue or of isolated intracellular particulates markedly alters the elution patterns of RNase. This change may be ascribed either to changes in the charge distribution of the active RNase itself, or to alterations in protein-protein interactions which affect the over-all charge of the complex. In this connection, the similarity between these results and those of Hirs *et al.*[7] should be emphasized. These workers observed a marked difference in RNase elution pattern between phosphate extracts and H_2SO_4 extracts of beef pancreas. Thus, while there is little doubt that RNase *A* of beef pancreas constitutes a homogeneous protein, both their results and those of this work suggest that the protein has somehow been altered from its intracellular condition. The isolation of a highly purified RNase from a phosphate extract of beef pancreas, in conjunction with a physicochemical analysis, would permit a comparison with RNase *A* and would contribute valuable information to this aspect of enzymology.

The observations concerning the activation of mouse pancreas RNase by a wide variety of treatments also may be of considerable physiological significance. The data suggest the presence of an inhibitor that is inactivated or removed by the chemical treatments. Direct data to support such a hypothesis are lacking in the pancreas, although Roth[16] has separated an RNase inhibitor from rat liver extracts. The coexistence of large amounts of RNase and of its substrate RNA, not only in the same tissue but in the same intracellular fraction, is reminiscent of the classic question, "Why doesn't the stomach digest itself?" An RNase inhibitor or an inactive form of the enzyme inside the cell can be postulated as a logical necessity.

Phosphate extracts of mouse pancreas or its intracellular fractions have been shown to produce different RNase chromatographic elution patterns from H_2SO_4 extracts. These data are related to the general question of the status of enzymes *in situ*. Although results on this problem are still too rudimentary to justify any generalizations, certain studies clearly demonstrate that the properties and activity of a number of isolated enzymes have been altered from their intracellular counterparts. The interesting work of Kaplan and Paik[17] on yeast catalase, in which they offer an explanation of the von Euler effect,[18] is one of the few systematic studies in this field. Kaplan and Paik postulate that the enzyme in the intact cell is present in an adsorbed, inhibited state.

Citri[19] has discovered 2 antigenically distinct penicillinases in *B. cereus* and has demonstrated a reversible transition between the 2 forms of the enzyme. Another significant contribution to this aspect of enzymology has been made by Swartz *et al.*[20] These workers have discovered a heat-activated DPN pyrophosphatase and a 5′-nucleotidase in *Proteus vulgaris* that are ordinarily present in an inhibited state. They have isolated a heat-labile inhibitor for the former enzyme from sonicates of the organism; many other examples of enzyme-inhibitor relationships are cited in their paper. Thus, an explanation of the effects of the various treatments on the elution patterns of RNase, as well as on its activity, will probably require additional studies of this type.

Summary

Homogenates of mouse pancreas have been separated by differential centrifugation into a nuclear-dense zymogen granule and 8 cytoplasmic fractions. The RNase activity of each fraction was determined before and after treatment with cold 0.25 N H_2SO_4. The acid treatment resulted in a large increase in RNase activity in the supernatant solution.

Each intracellular fraction, as well as the original homogenate, was chromatographed directly or after treatment with H_2SO_4. The acid-treated solutions produced RNase elution patterns very different from those of the phosphate extracts. Three activity peaks were found in the nonacid-treated homogenates, but only 2 were found in those subjected to H_2SO_4. In addition to these effects of acid, other agents were capable of solubilizing the microsomal-bound RNase and of activating the RNase of the supernatant solution.

The role of inhibitors in regulating intracellular enzyme activity and the possibility of alterations in proteins induced by the methods of extraction and isolation are discussed.

Acknowledgment

We thank Robert Holtzer for the data on the nitrogen analyses, and Allan Morris and K. Trupin for data included in FIGURE 6.

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PURIFICATION AND CHARACTERIZATION OF RIBONUCLEASE OF CALF SPLEEN

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Several intracellular ribonuclease (RNase) activities have been described[1-5] since it was established[6] that the RNase of spleen and thymus differed from the crystalline pancreatic RNase. The procedures used have provided RNase preparations that exhibit characteristic substrate specificities and that differ in their *p*H optima on ribonucleic acid (RNA), their ability to attack purine linkages, their magnesium ion requirements, and their heat stability. The spleen phosphodiesterase activities described by Heppel and Hilmoe[3] and Maver and Greco[4] have many characteristics in common, although the methods used differ markedly. Both preparations degrade RNA, "core", and the benzyl esters of ribonucleotides to yield exclusively the nucleoside-3′-phosphates, and the 2 enzyme preparations hydrolyze the cyclic purine and pyrimidine nucleotides to form nucleoside-2′-phosphates, but the Maver and Greco preparation also forms some nucleoside-3′-phosphate. Both are more sensitive to heating than the pancreatic RNase, and their activity on RNA is inhibited by magnesium ions at their *p*H optimum of 5.6 to 5.8.

With a very different method of purification, Kaplan and Heppel[5] have prepared a spleen RNase that closely resembles the pancreatic RNase in its catalytic properties and heat stability. RNA is not completely hydrolyzed, and only pyrimidine mononucleotides are formed. This spleen RNase differs from the pancreatic RNase only in its more acid *p*H optimum on RNA and in its behavior on the cation exchange resin XE-64. These investigators found that approximately 70 per cent of the RNase activity of the homogenate was destroyed in an initial heating step in their purification procedure, which involved 10 min. heating at 60° C. at *p*H 3.5. Most of the activity of the Maver and Greco preparations would have been destroyed by this treatment.

The present work describes the RNase activities of fractions obtained when spleen nuclease preparations were subjected to chromatographic analysis on cellulose anion and cation exchangers. This report is an extension of preliminary experiments.[7]

Methods

The nuclease preparations. The procedure used to separate the spleen nuclease preparations has been published.[4] These preparations contained an active DNase, as well as the RNase activity. The specific RNase activity of these preparations was about 1000 times that of the homogenates. The amounts of cathepsin and acid phosphomonoesterase present were very small, only traces being detected when relatively large samples of the preparations were assayed.

The substrates. Highly polymerized rat liver RNA was prepared according to the method of Grinnan and Mosher.[8] Less than 0.6 per cent of the total absorption of the RNA substrate was soluble in ice cold 3 per cent perchloric

acid. The DNA substrate was highly polymerized calf thymus nucleate prepared according to Hammarsten[9] as modified by Taylor *et al.*[10]

The barium salts of cyclic adenylic and cyclic cytidylic acid* were converted to the sodium salts on XE-64 resin. Adenosine-2′-benzyl phosphate and adenosine-3′-benzyl phosphate were prepared according to the method of Brown *et al.*[11] The cytidine-2′-benzyl phosphate and the cytidine-3′-benzyl phosphate were prepared according to the method of Brown and Todd.[12]

Determinations of enzyme activities. The RNase and DNase activities were assayed as previously described.[4] They were determined by measuring, at intervals, the extinctions at 260 mμ of the nucleic acids and their digestion products, which were made soluble in cold perchloric acid by the enzymic activity. The first-order rate constant, K_o, was calculated from the slope obtained by plotting against time, on semilogarithmic paper, the difference between the final extinction reading, E_f, and the extinction reading at time t, E_t. One unit of activity, K_o, is the amount required to hydrolyze in 1 hour 63 per cent of the substrate present.

The digestion mixture for the assay of the phosphodiesterase activities on the cyclic nucleotides contained 0.02 ml. 0.2 M sodium methylarsonate-acetate buffer at *p*H 6.6, 0.03 ml. cyclic nucleotide (50 μM/ml.), and 0.05 ml. of enzyme. The presence of relatively large amounts of phosphate in the eluting buffer precluded the use of microphosphorous determinations in the quantitative analysis of the products of hydrolysis of the cyclic nucleotides and of the benzyl esters of the nucleoside phosphates. The digests were chromatographed on paper using the techniques described by Brown *et al.*[11] After identification, the ultraviolet-absorbing regions of the paper were cut out and eluted. From their extinction at 260 mμ in a Beckman DU spectrophotometer the amount of isomer formed was determined.

Chromatography. Diethylaminoethylcellulose (0.9 mEq./gm.)[13] was prepared from 230 to 325-mesh cellulose obtained by sieving Solka-floc SW-40-B.† Carboxymethylcellulose (0.74 mEq./gm.)[13] was prepared from 100 to 230-mesh Solka-floc of the same type. These adsorbents, equilibrated with the appropriate starting buffers, were packed from dilute slurries under air pressure to form 1- × 52-cm. columns, the applied pressure being increased gradually as the column grew in length until a maximum of 10 psi was reached at about the time the column was completed.

In the experiment shown in FIGURE 2 (below), elution was accomplished by means of convex exponential gradients obtained with a mixer of the constant-volume type.[14] The gradients used in the experiments presented in FIGURES 3, 4, 6, and 8 (below) were produced by a variable-gradient mixer to be described in detail elsewhere.[15] In these experiments the mixer consisted essentially of a series of 5 identical mixing chambers in hydrostatic equilibrium and filled with the buffers specified in the captions.

Results

Preliminary chromatographic fractionation of the spleen nuclease preparations indicated that several RNase activities were present. These activities

* Purchased from Schwarz Laboratories, Mt. Vernon, N. Y.

† Product of Brown Co., Berlin, N. H.

were characterized by their *p*H optima on RNA, by the products released upon hydrolysis of the cyclic nucleotides, and by their activities after heating.

FIGURE 1 shows the effect on subsequent RNase activities at different *p*Hs of heating a spleen nuclease preparation at *p*H 3.5 for 15 min. at 70° C. and for 10 min. at 80° C. Less than one fourth of the acid RNase activity remained after heating, while the alkaline RNase activity was not changed.

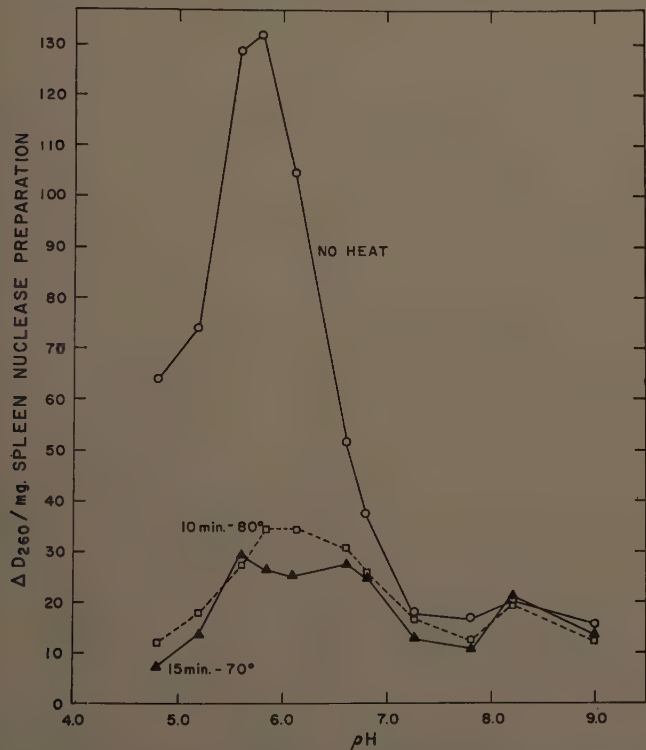

FIGURE 1. Effect of heat upon RNase activity at different *p*Hs. The increase in absorption at 260 mμ of the acid-soluble products of the digestion of rat liver RNA by 1 mg. of a spleen nuclease preparation. Assays were made at the different *p*Hs before and after heating the preparation at *p*H 3.5 for 10 min. at 80° C. and for 15 min. at 70° C. Assay digestion time was 20 min. at 24° C.

This greater sensitivity of the acid RNase is one of the characteristics that differentiate it from the pancreatic RNase and the spleen RNase of Kaplan and Heppel.[5]

None of the RNase fractions obtained hydrolyzed the 2′-benzyl-phosphate esters of the nucleosides, while all of these fractions hydrolyzed the 3′-isomers.

The result of a chromatographic fractionation of 200 mg. of a calf-spleen nuclease preparation on a DEAE-SF column is shown in FIGURE 2. The activities not retained on the column in 0.005 M sodium phosphate at *p*H 8.0 appear in the first peak, *A*. This fraction contained several enzyme systems that had contaminated the major acid RNase activity of the preparation. All

of the catheptic activity that had not been entirely removed by the initial separation procedure was localized in Fraction *A*. In this experiment most of the DNase activity preceded the RNase activity of the fraction. The chromatographic fractionation and properties of the DNase activities will be reported in a subsequent paper.

Most of the RNase activity in Fraction *A* was found in 1 tube. When hydrolyzing RNA, this fraction exhibited *p*H optima at 6.2 to 6.6 and at 8.0.

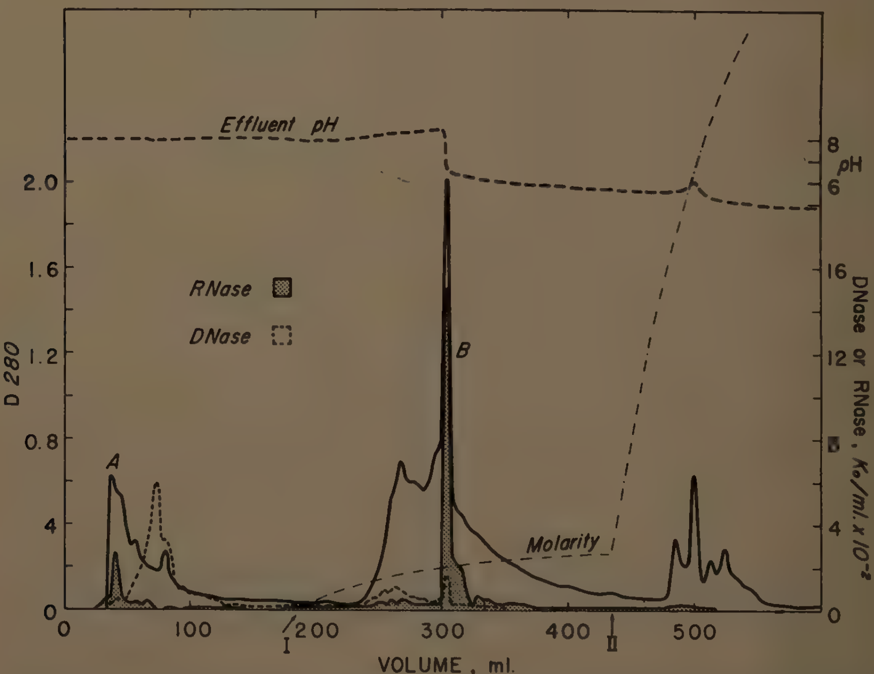

FIGURE 2. Spleen nuclease preparation (200 mg. in 2 ml.) chromatographed on DEAE-SF column (1 × 52 cm.). Initial buffer: 0.005 M Na phosphate, *p*H 8.0. A gradient to 0.15 M Na phosphate, *p*H 5.4, was begun at I; at II the gradient limit was changed to 2 M NaCl. A constant-volume (100 ml.) mixing chamber was used. Flow rate: 8 ml. per hour; with effluent collected in 4-ml. fractions.

Further evidence of more than one phosphodiesterase in this fraction was seen when the cyclic purine and pyrimidine nucleotides were hydrolyzed to yield the 2′-nucleotides and adenosine and cytidine. The presence of ribosides in the digests suggested the presence of a 3′-nucleotidase that liberated the phosphate group from the 3′-nucleotides and not from the 2′-nucleotides. The 3′-nucleotidase activity of this fraction was demonstrated further by its ability to form nucleosides from the 3′-nucleotides, but not from the 2′-nucleotides. A similar 3′-nucleotidase, found in germinating rye grass and barley by Shuster and Kaplan,[16] also specifically catalyzes the hydrolysis of 3′-nucleotides to nucleosides and inorganic phosphate.

An acid phosphomonoesterase active on disodium *p*-nitrophenyl phosphate

was shown to be present in Fraction *A* when assayed according to Koerner and Sinsheimer.[17] The significance of this activity in nucleotide metabolism is not clear.

A gradient of sodium phosphate from 0.005 M (*p*H 8.0) to 0.15 M (*p*H 5.4) effected the elution of another RNase peak, *B* (FIGURE 2). This fraction demonstrated a *p*H optimum of 5.6 to 5.8 on RNA, and the ability to hydrolyze the cyclic nucleotides to yield only the 2′-nucleotides. Fraction *B* represents

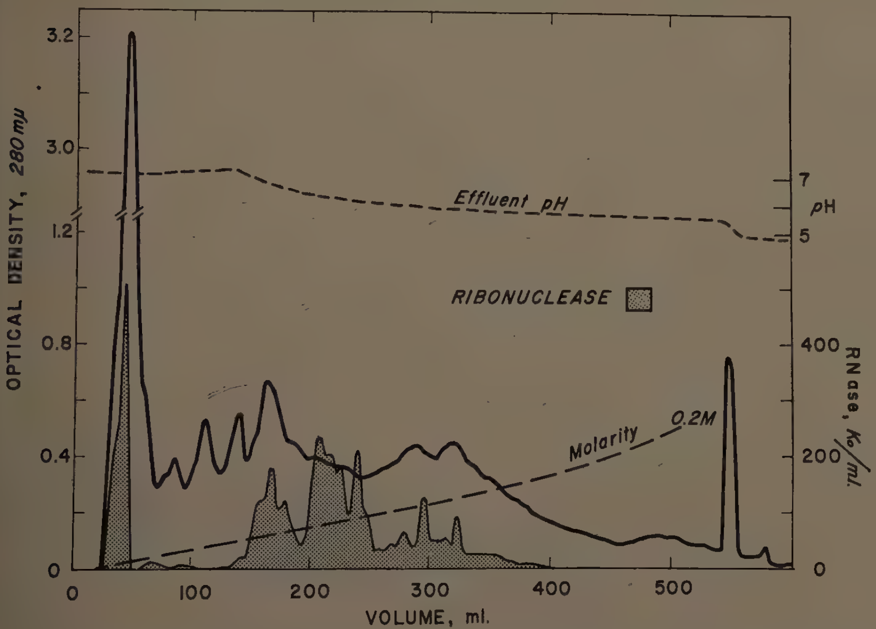

FIGURE 3. Spleen nuclease preparation (280 mg. in 4 ml.) chromatographed on DEAE-SF column (1 × 52 cm.). Initial buffer: 0.005 M Na phosphate, *p*H 7.0. Gradient to 0.2 M Na phosphate, *p*H 5.5, was begun immediately. Five identical mixing chambers filled with mixtures of the initial and final buffers were connected in series, and hydrostatic equilibrium was maintained while liquid was pumped from the first chamber to the column. Volume percentage of final buffer in the solutions: 0, 20, 40, 60, 100. Wash with 2 M NaCl was begun at arrow. Flow rate: 18 ml. per hour; with effluent collected in 4.6-ml. fractions.

most of the RNase activity of the calf spleen preparations, as well as of the spleen homogenates. The specific RNase activity in the peak tube was 5740 as compared to 1021 for the spleen nuclease preparation and an average of 1.07 for spleen homogenates. The abrupt emergence from the column of this RNase fraction, which was accompanied by a sharp drop in the *p*H of the effluent, suggested that a more gradual elution schedule was needed to fractionate further the RNase activity of this peak.

The results of a more gradual elution by means of a single gradient were seen when 200 mg. of a calf spleen nuclease preparation were put on a DEAE-SF column with 0.005 M sodium phosphate buffer at *p*H 7.0 (FIGURE 3). Again, the fraction not retained by the column appeared in the first 40 ml. of effluent.

This was followed by several peaks of RNase activity, which corresponded to Peak *B* of the preceding experiment, with *p*H optima at about 5.6 and the ability to hydrolyze the cyclic nucleotides to the 2′-nucleotides.

In order to obtain more material for rechromatography, 500 mg. of another spleen nuclease preparation was applied to a DEAE-SF column and chromatographed under the same conditions (FIGURE 4). Fraction *A* hydrolyzed cyclic adenylic acid to give 70 per cent 2′-nucleotides and 29 per cent 3′-nucleotides as calculated from the adenosine in the digests. Only 2′-nucleotides were found in the digests of the cyclic adenylic and cytidylic acids by the peak

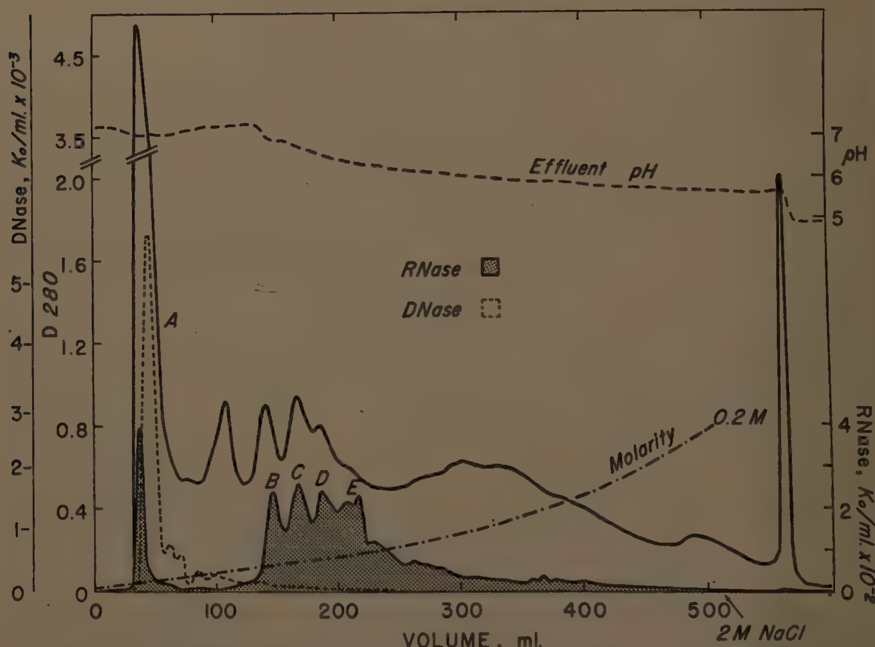

FIGURE 4. Spleen nuclease preparation (500 mg. in 4 ml.) chromatographed on DEAE-SF column (1 × 52 cm.). Conditions are as in FIGURE 3, except for difference in proportions of buffers charged to gradient mixer: 0, 20, 30, 50, and 100 volume per cent of final buffer.

activities in fractions *B*, *C*, *D*, and *E*. The *p*H-activity curves in FIGURE 5 show that Fraction *A* contained a concentrated mixture of RNase activities, with higher *p*H optima than the RNase in fractions *B*, *C*, *D*, and *E*.

There were also differences in stability when these RNase fractions were heated at 70° C. for 15 min., as may be seen in TABLE 1. These rates of activity, before and after heating, were determined at *p*H 5.8. On the other hand, when the RNase in Fraction *A* was assayed at *p*H 8.2, before and after heating, it was found that 85 per cent of its original activity remained after heating.

Only the more heat-stable activity in Peak *B* and the slight differences in *p*H optimum on RNA were found to differentiate the 4 acid RNase activities in fractions *B*, *C*, *D*, and *E*. The ratios of their activity on RNA to their

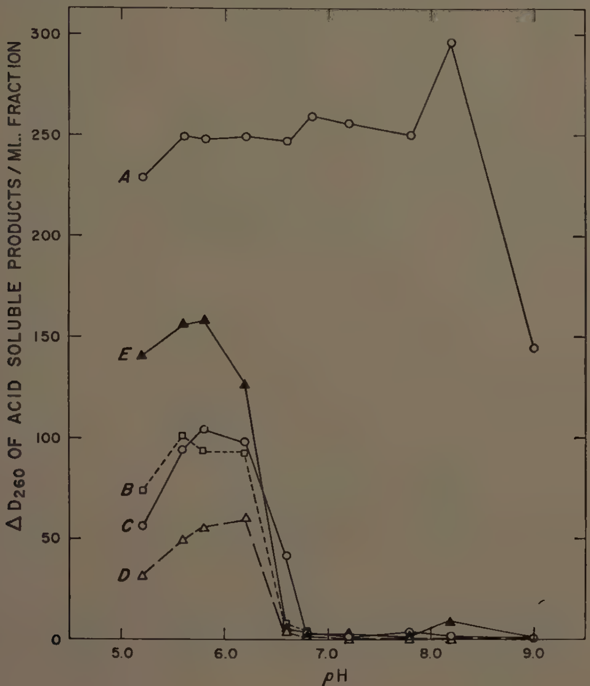

FIGURE 5. *p*H-Activity curves of the peak RNase activities eluted from DEAE-SF. The increase in absorption, at 260 mμ, of the acid-soluble products of digestion of RNA by 1 ml. of eluent at different *p*Hs was determined after 20 min. digestion at 24° C. Activities are given for fractions *A*, *B*, *C*, *D*, and *E*, shown in FIGURE 4.

TABLE 1

COMPARISON OF THE NUCLEASE PREPARATION AND FRACTIONS ELUTED FROM DEAE-SF WITH RESPECT TO THEIR HEAT STABILITIES AND THE RATIOS OF THEIR ACTIVITY ON RNA TO THEIR ACTIVITY ON CYCLIC ADENYLIC ACID

	Percentages of initial RNase activity after heating	Activity on RNA† / Activity on cyclic adenylic acid
Nuclease preparation*	22.7	—
Fraction *A*	39.7	—
Fraction *B*	36.1	2.42
Fraction *C*	16.8	2.93
Fraction *D*	11.7	3.21
Fraction *E*	14.9	2.90

* The nuclease preparations before chromatography and the fractions in FIGURE 4 were heated at the *p*H (7.0 to 6.5) of the fractions for 15 min. at 70° C. and, after dilution were assayed at *p*H 5.8.

† Ratio of acid RNase activities on RNA to their activities in percentage hydrolysis of cyclic adenylic acid to the 2′-nucleotide.

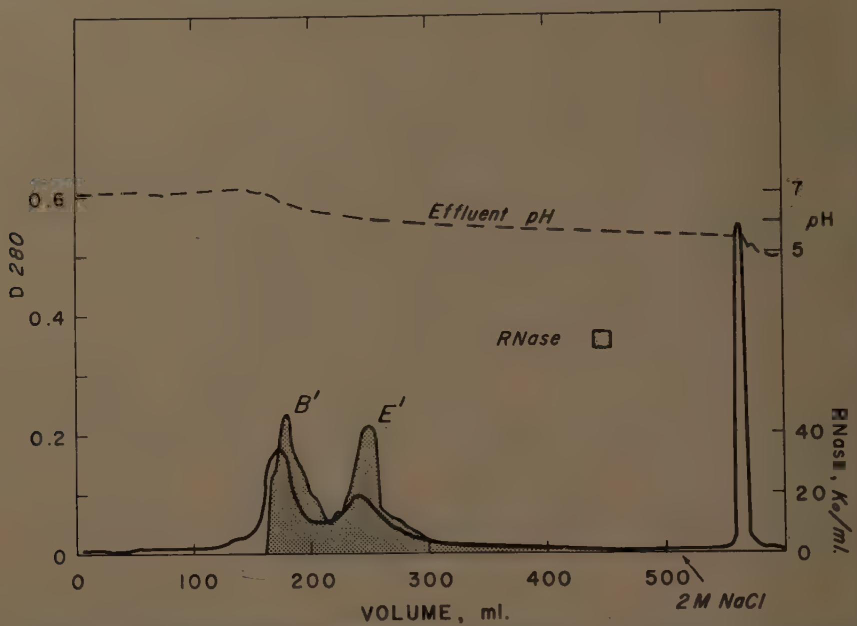

FIGURE 6. Rechromatography of peaks *B* and *E* of FIGURE 4 on DEAE-SF. Conditions are as in FIGURE 4.

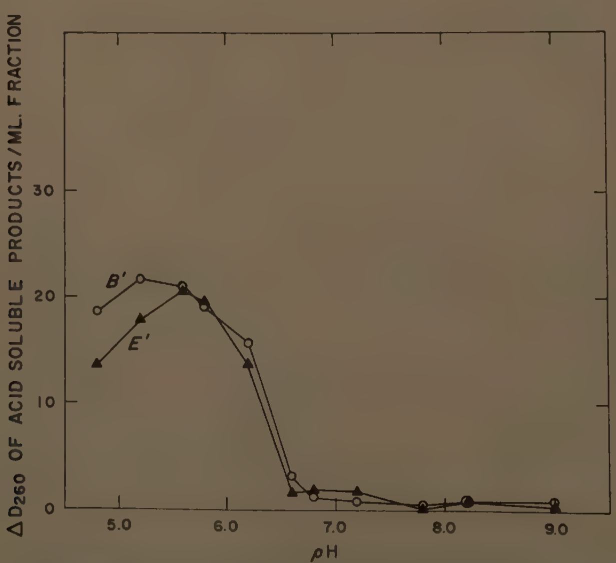

FIGURE 7. *p*H-Activity curves of 2 peaks of RNase activity after rechromatography on DEAE-SF. The increase in absorption at 260 mμ of the acid-soluble products of digestion of RNA by 1 ml. of eluent at different *p*Hs determined after 20 min. digestion at 24° C. Fraction *B* and Fraction *E* of FIGURE 4 are represented (after rechromatography) as Fractions *B'* and *E'*.

diesterase activity on cyclic adenylic acid, measured as percentages of hydrolysis to the 2′-nucleotide, showed small differences (TABLE 1). It is possible that a detailed chromatographic study of the products of digestion of RNA or "core" would reveal qualitative differences in the activities of these fractions. In order to establish the fact that the 4 acid RNase fractions contained different protein entities with similar RNase activities, the first and fourth fractions (*B* and *E*) were combined and rechromatographed on DEAE-SF under the same conditions as before. The result is shown in FIGURE 6. The chromatographic

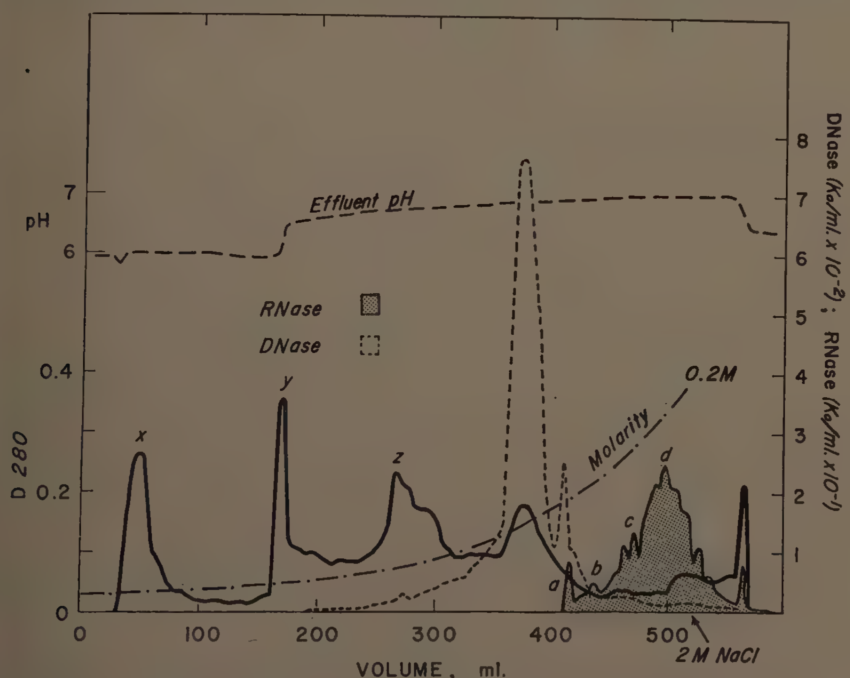

FIGURE 8. Rechromatography of Fraction *A* of FIGURE 4 on CM-SF (1 × 52 cm.). Initial buffer: 0.015 M Na phosphate, *p*H 6.0; final buffer: 0.02 M Na phosphate, *p*H 7.0. Buffers in mixing chambers: 0, 5, 10, 15, 100 volume per cent of final buffer. Other conditions are as in FIGURE 3.

validity of these peaks is thus verified, although their origin remains obscure. Aqvist and Anfinsen[18] reported a similar phenomenon, in which the microheterogeneity of RNase from sheep pancreas was demonstrated by chromatography on DEAE-SF columns.

All of the RNase activity of the two fractions, measured in K_o/ml., was recovered, and their respective ratios of activity on RNA to activity on cyclic adenylic acid remained the same after rechromatography. Fraction *B*′, which corresponded to the first of the 4 original acid RNase peaks, was more sensitive to heating after rechromatography, with only 15 per cent of its activity remaining after 15 min. at 70° C.—as compared to 36 per cent before rechromatography. Also, the *p*H-activity curves in FIGURE 7 show that the *p*H optimum

of this rechromatographed Fraction B' had changed from 5.6 to 5.2. The significance of the small changes in *p*H optima is unknown, although they may be related to dissociation of inert protein.

Fraction *A* of the nuclease preparation not retained on the anion exchanger was applied (FIGURE 8) in 0.015 M sodium phosphate at *p*H 6.0 to the cation exchanger, carboxymethyl-SF (CM-SF). Four considerably diluted RNase activities were found in peaks *a*, *b*, *c*, and *d*, and the total activity recovered amounted to 75 per cent of that applied to the column. The *p*H-activity curves in FIGURE 9 show that fractions *a* and *c* have RNase activity over a

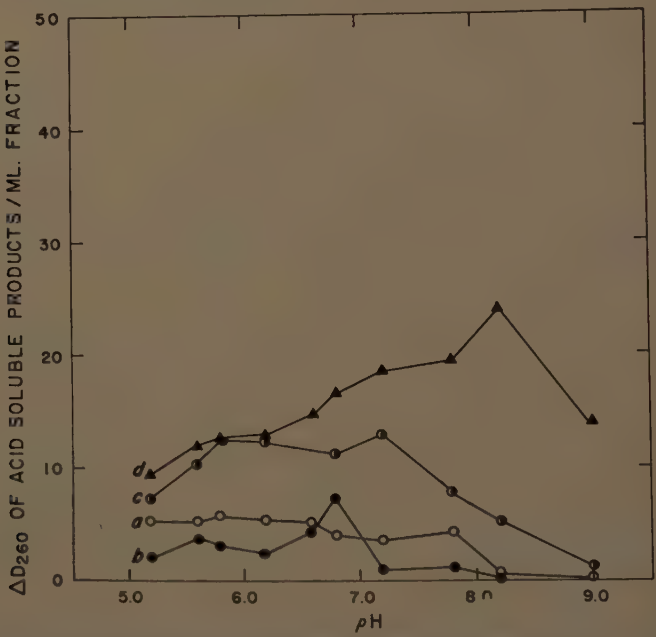

FIGURE 9. *p*H-Activity curves of the peak RNase activities eluted from CM-SF. The increase in absorption at 260 mμ of the acid-soluble products of digestion of RNA by 1 ml. of fraction at different *p*Hs, determined after 20 min. digestion at 24° C. Activities are given for 4 peaks—*a*, *b*, *c*, and *d*—obtained after rechromatography on CM-SF (FIGURE 8).

broad *p*H range. More definite *p*H optima were exhibited by Fraction *b* at pH 6.8 and Fraction *d* at *p*H 8.2, the latter retaining 79 per cent of its activity after being heated for 15 min. at 70° C. These fractions represent the RNase activities that contaminated the original nuclease preparations.

The phosphodiesterase activity responsible for hydrolyzing the cyclic nucleotides to the 3′-nucleotides was found in Peak *b* (FIGURE 8), whose *p*H optimum was 6.8. However, all the fractions rapidly hydrolyzed the cyclic nucleotides to yield the 2′-nucleotides. The ratios of activity on RNA to phosphodiesterase activity on the cyclic nucleotides were so low as to indicate the presence of a phosphodiesterase activity not related to RNase activity. Examination of the D_{280} peaks, *x*, *y*, and *z* (FIGURE 8), revealed that an active phosphodiesterase, which specifically formed the 2′-nucleotides when hydrolyzing the cyclic

nucleotides, was concentrated also in peaks *y* and *z*, where only traces of RNase activity could be found. As an impurity in the nuclease preparations, its identity would be masked by the acid RNase, which also liberates the 2′-nucleotides from the cyclic nucleotides. This enzyme has been found in similar experiments when Fraction *a*, which was not retained by the DEAE-SF, was rechromatographed on CM-SF. Davis and Allen[19] have reported the partial purification of a phosphodiesterase from beef pancreas, which yielded only the 2′-nucleotides in its action on cyclic purine and pyrimidine nucleotides. Their phosphodiesterase was obtained when a pancreatic preparation passed through a column of IRC-50 that had been equilibrated with 0.1 M acetate at *p*H 6.0; RNase was retained on the column.

Discussion

With appropriate elution gradients on DEAE-SF and CM-SF columns, the RNase and DNase activities of a spleen nuclease preparation have been separated. Unknown enzymes in the preparations were also separated so that they could be identified. Thus, a 3′-nucleotidase and a phosphodiesterase activity were found; the latter was not related to RNase and formed specifically the 2′-nucleotides from cyclic nucleotides.

The alkaline RNase activity that contaminated the major acid RNase activity in the preparations was separated and appears, from its *p*H optimum, heat stability, and magnesium ion activation, to be similar to the alkaline RNase of the liver preparations of Roth[2] and de Lamirande *et al.*[1] RNase activity similar to that of Kaplan and Heppel[5] was present in a very small amount and was characterized by its heat stability and by the formation of 3′-adenylic acid upon hydrolysis of cyclic adenylic acid.

The origin or significance of the microheterogeneity shown by the appearance of several proteins with similar RNase activities after application to DEAE-SF poses a problem for future studies. It is interesting to note that the DNase protein did not show this phenomenon. Whether the individual peaks represent molecules with structural differences established during their synthesis or whether they arose as a result of metabolic or chemical degradation thereafter cannot be decided at this time. There is, moreover, the possibility that some of the peaks are a result of complex formation with other proteins.

Summary

The RNase activities have been separated from the DNase activity in spleen nuclease preparations by means of appropriate chromatographic techniques on columns of diethylaminoethylcellulose. Several acid RNase fractions with similar characteristics were eluted; their chromatographic validity was established by rechromatography of two of the peaks.

The heat-stable alkaline RNase of spleen was also separated by chromatography on carboxymethylcellulose.

A 3′-nucleotidase and a phosphodiesterase, which was free of RNase activity and specifically hydrolyzed the cyclic nucleotides to the 2′-nucleotides, were found as impurities of the nuclease preparations. These enzymes were not retained on the diethylaminoethylcellulose, but could be chromatographed on carboxymethylcellulose.

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COMPARATIVE STUDIES ON TISSUE RIBONUCLEASES*

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It is becoming abundantly clear that an enzyme type that is widespread in nature may occur in many diverse structures. It is entirely possible that an enzyme such as ribonuclease (RNase), which has been found in many animal, plant, and microbiological forms, is constructed differently by each form. Differences in enzyme structure among the different species may be slight, consisting of only one or a few alterations in amino acid kind or arrangement; or they may be considerable, involving completely different primary, secondary, and tertiary structures of the enzyme protein. Divergences in structure would be reflected of course, in divergences in enzyme properties, and it is already apparent that enzymes isolated from some sources (for example, rat liver and calf pancreas) resemble each other closely in properties and action, while others seem to have in common only the ability to degrade ribonucleic acid (RNA). The picture is complicated further by the fact that there is good evidence that some animal tissues contain several different kinds of RNase; the purpose of this complex arrangement remains an intriguing question.

It is a problem of more than academic interest to isolate, purify, and compare the RNases from different sources; eventually, to the extent of determining amino acid sequence, as well as aspects of the cross linkages of peptide chains, hydrogen bonding, and the three-dimensional structure of the macromolecule. Such studies can yield much useful information, not only concerning the mechanism of action of the particular enzyme but, in the larger sense, concerning the more general aspects of hydrolytic enzyme action. It is important to know, therefore, whether the many diverse RNases contain some common denominator of structure responsible for the particular accomplishment of this enzyme. Since different RNases degrade RNA in different ways, a correlation of the mode of degradation with structure would be a further refinement of our understanding regarding the mechanism of action of enzymes. Equally important to these problems is the determination of the function of the enzyme within the cell, and it is here that comparative studies may prove of considerable value. Correlation of the biochemical characteristics of the cell with the properties of the enzyme may yield valuable clues as to function. An example of this is the finding by several investigators[1, 2] that there appears to be a direct correspondence between RNase activity and the rate of cell growth and development.

In view of the apparent multiplicity of RNases in nature, it undoubtedly would be helpful if some system of classification were devised. However, our information concerning all but a few RNases is so scanty as to make this difficult at the present time. As a beginning, TABLE 1 lists a few of the enzymes and suggests one way in which they might be classified—that is, according to

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the optimum pH; a better classification might be according to the bonds of RNA that are attacked. Obviously, many other methods could be used. The rather incomplete list of TABLE 1 illustrates well the great diversity to be found in RNases.

In this report we shall be concerned primarily with the RNases of rat liver and an RNase isolated from the protozoan *Tetrahymena pyriformis* strain W; these are compared with crystalline pancreatic RNase.

TABLE 1
SOME PROPERTIES OF RNASES FROM DIFFERENT SOURCES

Source	pH optimum	Heat stability*	—SH dependence	RNA products*	References
Bovine pancreas	7.8	high at $pH < 7$	0	pyrimidine-3′-nucleotides	8, 9
Rat mitochondria	7.8	high	0	pyrimidine-3′-nucleotides	6, 14
Rat supernatant fraction	7.6	high	0	—	21
Bovine spleen	6.5	high (at low pH)		pyrimidine-3′-nucleotides	4
Rat spleen mitochondria	~7.0	—	0	—	18
Squid gill	~7.0	high at low pH (0 at pH 7.6)	high	—	15
Castor bean	5.0	high	—	—	13
Rat liver lysosomes	5.8	low	0	pyrimidine and purine nucleotides	14 and this paper
Tetrahymena	5.5	moderate	0	pyrimidine and purine nucleotides	15 and this paper
Tobacco leaf	5.1	high	0	pyrimidine and purine-3′-nucleotides	11, 12
Rye grass	4.5	high	0 (inhibited by —SH)	pyrimidine and purine-5′-nucleotides	10

* Dash indicates data not available.

It appears that rat liver may contain at least three different RNases. One, optimally active at pH 7.8, is present in the mitochondria; another, optimally active at pH 5.8, is also found in the mitochondrial fraction but, according to de Duve,[3] is contained within the lysosomes that separate with the mitochondria. The third rat liver RNase is present in an inactive state in the supernatant fraction and is bound to an RNase inhibitor, from which it may be released by various treatments.[5,7]

Materials and Methods

The methods of preparation and purification of the RNases and RNase inhibitor from rat liver have been described.[6,7,21] *Tetrahymena* RNase was obtained from 3-day-old cultures. The cells were thoroughly washed, con-

centrated by gentle centrifugation, and then homogenized in a Logeman colloid mill. The homogenate was centrifuged in a Spinco preparative ultracentrifuge at 60,000 g for 60 min.; the supernatant fluid, which contained most of the RNase activity, was used for the enzyme assays. This procedure purified the enzyme about tenfold.

The assay system for all the experiments utilized 0.5 ml. of acetate, borate, cacodylate buffer[19] of the appropriate *p*H, 0.5 ml. of a 1 per cent solution of a commercial preparation of RNA,* and 0.5 ml. of enzyme and other test components or water. This mixture was incubated for half an hour at 37° C. and then precipitated with 1.5 ml. of acid-alcohol.[16] After standing briefly, the mixture was filtered through 7-cm. circles of Whatman No. 42 filter paper, and the optical density of a 1:50 dilution of the clear filtrate was determined at 260 mμ. A standard containing 0.0075 γ of crystalline pancreatic RNase was always run simultaneously, and gave an optical density increase of from 0.100 to 0.110 under these test conditions; appropriate blanks also were run. In general, tissue blanks were negligible. One unit of enzyme activity is the amount of enzyme causing an increase in O.D. of 0.100 under the conditions described.

Adenosine-2′,3′-phosphoric acid (cyclic A) and uridine-2′,3′-phosphoric acid (cyclic U) were obtained commercially† as barium salts. They were converted to ammonium salts by passage through a column of Dowex 50 in the ammonium form, and the cyclic nucleotides were obtained in a final concentration of approximately 2 mg./ml.; when freshly prepared, they were chromatographically pure. Generally, 0.1 ml. of buffer, 0.1 ml. of substrate, and 0.05 ml. of enzyme (containing from 1 to 5 units of enzyme activity) were incubated at 37° C. for 1 hour, and 25-μl. samples were removed at 10 and 60 min. for spotting; no hydrolysis of the substrate occurred in buffer alone under the same conditions. The spots were developed in isopropanol-water-ammonia solvent described by Markham and Smith.[20] The appearance of a slower-migrating nucleotide in the enzyme-treated samples was taken as evidence of hydrolysis of the cyclic nucleotide. In experiments with polyadenylic acid (poly A) and polyuridylic acid (poly U), run similarly, the controls in the above solvent system did not migrate, and disappearance of the spot from the origin was taken as evidence of hydrolysis.

Antiserum to crystalline pancreatic RNase was prepared by injecting 2 mg. of the crystalline enzyme dissolved in 0.5 cc. of saline into the ear vein of rabbits 3 to 4 times weekly for 4 weeks.

Results

One of the first factors investigated was the response of the various enzyme preparations to certain specific RNase inhibitors. Some of the results obtained are shown in TABLE 2.

Crystalline pancreatic RNase, in confirmation of the results of Bernheimer and Steele,[17] was quite susceptible to the action of the lilac leaf inhibitor. Inhibition varied from 77 to 100 per cent in a series of 4 experiments. *Tetra-*

* Product of Schwarz Laboratories, Inc., Mt. Vernon, N. Y.; occasionally, other RNA preparations were used.

† Product of Schwarz Laboratories, Inc.

hymena RNase was also considerably inhibited by this agent, but both rat liver alkaline RNases were little affected, although small inhibitions were registered in some experiments. Acid RNase of rat liver was moderately inhibited by the plant extract.

The results with rabbit antiserum, which showed reaction only with crystalline pancreatic RNase, would appear to indicate that in this case there is not enough of a similarity in the RNases from two different species to give cross reactions with the antiserum prepared to one of them. This does not preclude the fact, however, that there may be considerable structural similarities in different enzymes. In the last column of TABLE 2 are listed the responses of

TABLE 2
THE EFFECT OF INHIBITORS ON VARIOUS RNASE PREPARATIONS*

Preparation	Lilac leaf inhibitor		Rabbit antiserum to crystalline pancreatic RNase		Rat liver supernatant RNase inhibitor	
	Amount of inhibitor ml.	Average inhibition, per cent	Amount of inhibitor ml.	Average inhibition, per cent	Amount of inhibitor ml.	Average inhibition, per cent
Crystalline pancreatic RNase	0.05	89.2	0.1 (1:10 dilution) 0.1 (1:50 dilution)	98 19	0.05‡	90
Alkaline RNase of rat liver supernatant fraction	0.05	13.9	0.1 to 0.7	0	0.05	90
Alkaline RNase of rat liver mitochondria	0.05	4.1	0.1 (1:10 dilution) 0.3 (1:10 dilution)	0	0.05	80
Acid RNase of rat liver	0.05	34.0	0.1 to 1.0 (1:10 dilution)	0	0.05	25
Tetrahymena RNase†	0.05 0.10 0.15	39.6 64.8 100.	0.1 to 1.0 (1:10 dilution)	0	0.05	0

* Using 1 unit of enzyme activity.
† With 2 to 5 units of RNase activity.
‡ Of unpurified supernatant fraction.

the enzymes to the RNase inhibitor found in rat liver supernatant fraction. Since this inhibitor exhibits little activity in the *p*H range 5 to 6, enzymes having their *p*H optima in this range are not greatly affected.

The effect of cupric ion on the RNases is shown in FIGURE 1. It is somewhat surprising that Cu^{++} is markedly inhibitory to some of the enzymes, while Pb^{++} has very little effect on any of them, even at relatively high concentrations. Crystalline pancreatic RNase appears most susceptible to the inhibitory action of Cu^{++}. *Tetrahymena* RNase and the alkaline RNase of mitochondria are also strongly inhibited, but to a somewhat lesser degree than the pancreatic enzyme. Alkaline RNase of the supernatant fraction appears much less susceptible to the action of cupric ion, the highest concentration used giving only 65 per cent inhibition. Acid RNase, which is not shown, is not seriously

affected by low concentrations of copper, and the curve for its response to cupric ion falls below even that of alkaline RNase of supernatant.

Finally, the activity of the various enzyme preparations against certain specific substrates has been examined; these results are shown in TABLE 3. Also shown in this table are the results of assays of the enzyme preparations for phosphatase and phosphodiesterase activity.

Preparations of both the acid RNase of rat liver and *Tetrahymena* RNase contained considerable phosphatase activity. In some recent purifications, it has been possible to remove most of this phosphatase from the *Tetrahymena* enzyme. None of the preparations had any appreciable phosphodiesterase

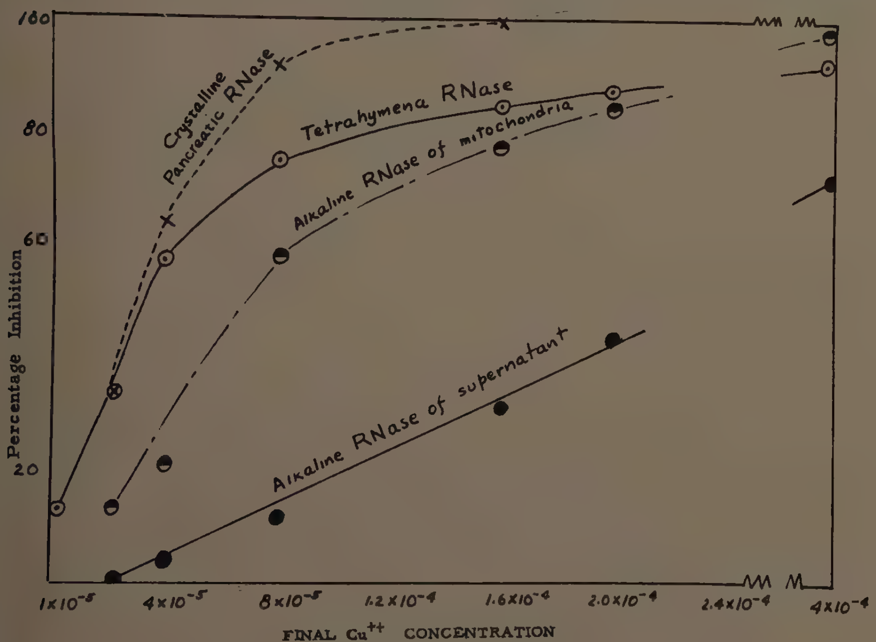

FIGURE 1. The effect of cupric ion on various RNase preparations.

activity when tested against calcium bis-(p-nitrophenyl) phosphate. Considering next the activities against the specific substrates: *Tetrahymena* RNase rapidly hydrolyzed both poly A and poly U, and also hydrolyzed cyclic A and cyclic U. It has not been determined whether the 2′ or the 3′ isomer of the nucleotides is obtained. The action of acid RNase of rat liver was similar to that of *Tetrahymena* RNase; both poly A and U and cyclic A and U were hydrolyzed. In this case the poly A and cyclic A appeared to be attacked at a slower rate than with *Tetrahymena*. This may have been due to the lower specific activities of the acid RNase preparations used. These results with acid RNase are in agreement with those reported recently by Nodes and Reid[14] and others.[22] Alkaline RNase of rat liver mitochondria hydrolyzed cyclic U but not cyclic A, and had no appreciable action against either poly A or poly U. This inactivity against poly U is rather surprising. Possibly, longer times of

incubation than the 1 hour used would demonstrate splitting of poly U by this enzyme. When one compares the alkaline RNase of rat liver supernatant fraction with that of mitochondria, distinct differences in specificities become clear. The alkaline RNase of the supernatant fraction had no action against the cyclic substrates under the conditions used, while, in contrast to the alkaline RNase of mitochondria, it readily split poly U. It may be seen from TABLE 3 (Column 2) that, with the exception of pancreatic RNase and the alkaline RNase of mitochondria, the enzyme preparations are only partly purified. It is possible, therefore, that the activities observed are, in some cases, the effects of more than 1 enzyme. Further specificity studies on more highly purified preparations would be desirable. The *Tetrahymena* enzyme, it is believed, offers considerable promise: it is easily prepared and purified to a

TABLE 3
THE ACTION OF RNASE PREPARATIONS ON VARIOUS SUBSTRATES*

Preparation	Approximate purification factor	Phosphatase activity	Phosphodiesterase activity	Substrate			
				Uridine-2′,3′-phosphate	Adenosine-2′,3′-phosphate	Poly A	Poly U
Crystalline pancreatic RNase	Crystalline	0	0	+	0	0	+
Alkaline RNase of rat liver supernatant fraction	20	0	Slight	0	0	0	+
Alkaline RNase of rat liver mitochondria	1000	0	0	+	0	0	0
Acid RNase of rat liver	6	+	Slight	+	+ Slow	+ Slow	+
Tetrahymena RNase	10	+	0	+	+	+	+

* Key: +, hydrolysis; 0, no appreciable hydrolysis.

high specific activity, it is moderately stable, has rather broad specificity, and may be useful in the preparation of specific nucleotides. Also, if it can be obtained in the crystalline state, it would be of considerable interest to compare its structure and properties with those of crystalline bovine pancreatic RNase.

Acknowledgment

The lilac leaf RNase inhibitor used in these studies was supplied by A. W. Bernheimer. We are also indebted to Leon Heppel for samples of poly A and poly U.

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LIVER RIBONUCLEASES*

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Pioneer work establishing the fact that liver contains both an acid and an alkaline ribonuclease (RNase) is referred to elsewhere in this monograph (de Lamirande and Allard, 1958; Roth, 1958) and need not be recapitulated here. Our own investigations on cytoplasmic RNases in rat liver have dealt with three main topics: specificities, intracellular distributions, and changes in levels under the influence of hormones or carcinogenic azo dye.

At the outset of our work there was no published information on specificities, but it was known that both acid and alkaline RNase are present in mitochondrial fractions (de Lamirande *et al.*, 1954; Roth, 1954, 1957), and that alkaline RNase occurs also in supernatant fractions—but in association with an inhibitor (Roth, 1957). However, it could not be assumed that RNases are indeed constituents of mitochondria. Results of meticulous studies from de Duve's laboratory (de Duve *et al.*, 1955) have suggested that acid RNase and certain other hydrolases are associated, not with mitochondria, but with acid phosphatase-containing granules ("lysosomes"); such enzymes may not show full activity in the assays unless a treatment designed to disrupt the granules is first applied.

Assays. In the usual assay procedure (de Duve *et al.*, 1955; Stevens and Reid, 1956), with RNA as substrate, activities were assessed from the increase in the soluble ultraviolet-absorbing (260 mμ) material remaining after incubation and final addition of perchloric acid-uranyl acetate reagent. Experimental tubes were incubated for 31 min. at 37° C., and blanks (containing both tissue sample and RNA) for 1 min. Incubation was carried out in *p*H 5.0 acetate with yeast RNA for acid RNase, and in *p*H 8.0 histidine with liver RNA for alkaline RNase. With the latter enzyme, despite the presence of RNA in high concentration (de Lamirande *et al.*, 1954), the apparent activity declined sharply when the E_{260} increase exceeded a low limit (0.1 under our conditions).

Recently (Nodes, 1958), on the basis of findings presented below, a paper-chromatographic assay method has been developed using cyclic nucleotides as substrates; this method estimates acid RNase in tissues without interference by alkaline RNase.

In all our assays, full liberation of the activity in particles has been ensured by an initial freeze-thaw procedure in hypotonic medium. Moreover, samples to be assayed for alkaline RNase have usually been heated at 60° C. for 10 min. at *p*H 3.5; this procedure (Kaplan and Heppel, 1956) destroys not only the supernatant-fraction inhibitor, but also acid RNase.

Liberation of activity from particles. Acid RNase is known to resemble acid

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phosphatase in the extent to which the activity is liberated from particles by various disruptive treatments (de Duve *et al.*, 1955). We have now found that, on carrying out repeated freezing and thawing in an isotonic sucrose medium (Gianetto and de Duve, 1955), alkaline RNase is liberated even less readily than acid phosphatase (FIGURE 1). Since a similar high resistance to liberation under these conditions has recently been found (Bendall, 1958) with glutamic dehydrogenase, a truly mitochondrial enzyme, no conclusions can be drawn from such experiments as to the intracellular location of RNases. These experiments, however, have found a useful application, as described below, arising from the further finding that recentrifugation of freeze-thawed particles sediments the alkaline RNase activity, but little of the acid RNase activity.

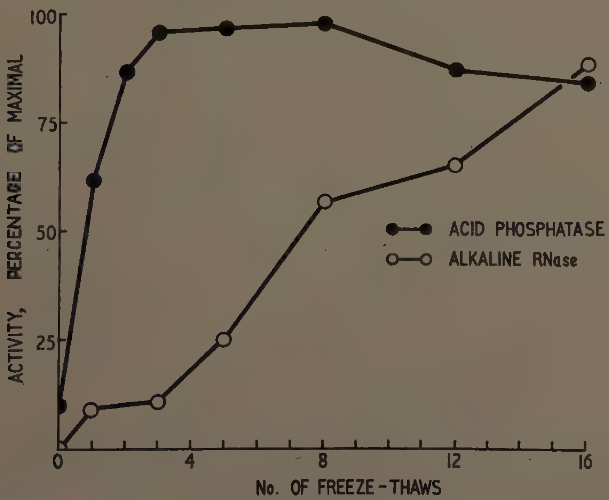

FIGURE 1. Liberation of acid-phosphatase and alkaline-RNase activities on subjecting a mitochondrial fraction to repeated freezing and thawing in isotonic (0.25 M) sucrose. Values for maximal activity were obtained by freezing and thawing 8 times in hypotonic sucrose.

Specificities

Enzyme preparations. The simple procedure shown in FIGURE 2 gives preparations of acid RNase and of alkaline RNase, each essentially free from the other, although more contaminated with extraneous protein than the preparations described by Roth (1957) and Zytko *et al.* (1958). After freeze-thawing a mitochondrial fraction in a hypotonic medium (usually 3 times), and after recentrifugation, a supernatant is obtained that serves as a satisfactory acid-RNase preparation; any slight contamination with alkaline RNase is, as will become evident, of no consequence for specificity studies. The debris contains alkaline RNase and, as will be shown, traces of acid RNase that are effectively removed by heat treatment at *p*H 3.5, preferably followed by exposure to H_2SO_4 at 0° C., as in the procedure of Roth (1957). These treatments have the further effect of rendering the alkaline RNase soluble.

With these enzyme preparations we have obtained information on specificities

(Nodes and Reid, 1958), which is presented below and is in gratifying agreement with that recently reported by Zytko *et al.* (1958).

Specificities with RNA as substrate. That cyclic nucleotides are intermediates in the breakdown of RNA by pancreatic RNase was demonstrated by Markham and Smith (1952), in whose experiments the RNA was digested in dialysis sacs, and the dialyzates concentrated for chromatographic examination. Similar experiments with acid RNase (FIGURE 3) have now shown that here, too, the dialyzate contains cyclic mononucleotides, as well as straight-chain mononucleotides (2′ and 3′ or both). An important difference from pancreatic RNase is that purine, as well as pyrimidine nucleotides (cyclic and straight-chain), are

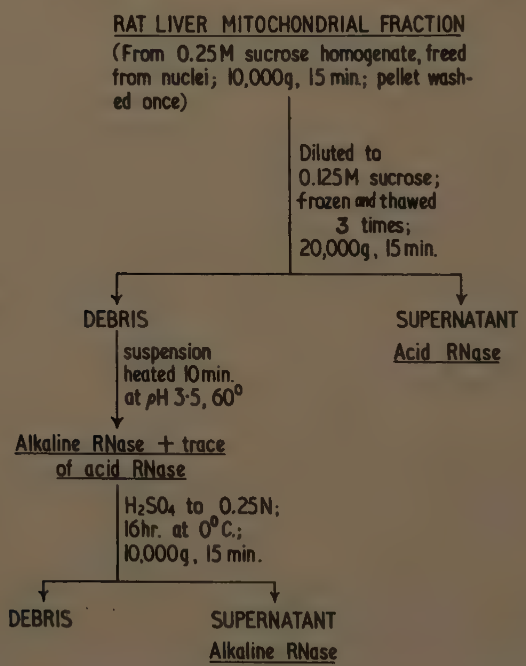

FIGURE 2. Method for obtaining preparations of alkaline RNase and of acid RNase.

formed, the identification of the various bands in FIGURE 3 having been confirmed by rechromatography in ammonium sulfate-sodium acetate-isopropanol.

When alkaline RNase is incubated with RNA, cyclic pyrimidine nucleotides are formed. Alkaline RNase, unlike acid RNase, does not attack the "core" that remains after exhaustive digestion of RNA with pancreatic RNase and is known to be rich in purine nucleotides (FIGURE 4).

Specificities with cyclic nucleotides as substrates. FIGURE 5 shows that cyclic cytidylic acid is attacked by acid RNase, to give 3′-cytidylic acid; some cytidine was also formed, presumably by the action of a contaminating phosphatase. With cyclic adenylic acid as substrate and the same solvent system (ammonium sulfate-sodium acetate-isopropanol), there is the difficulty that any adenosine formed by phosphatase action will run with the same R_f as 3′-adenylic acid.

As is shown in FIGURE 6, this difficulty was overcome by 2-dimensional chromatography—initially with butanol-water in which adenosine moves, but nucleotides remain at the origin; markers were then applied and chromatography carried out as for FIGURE 5. Since cyclic cytidylic acid had given 3′-cytidylic acid, we were surprised to find 2′-adenylic acid, but no 3′-adenylic acid. We have satisfied ourselves that the cytidine and adenosine shown in FIGURES 5 and 6 could not have been formed by complete breakdown of 1 isomer preferentially.

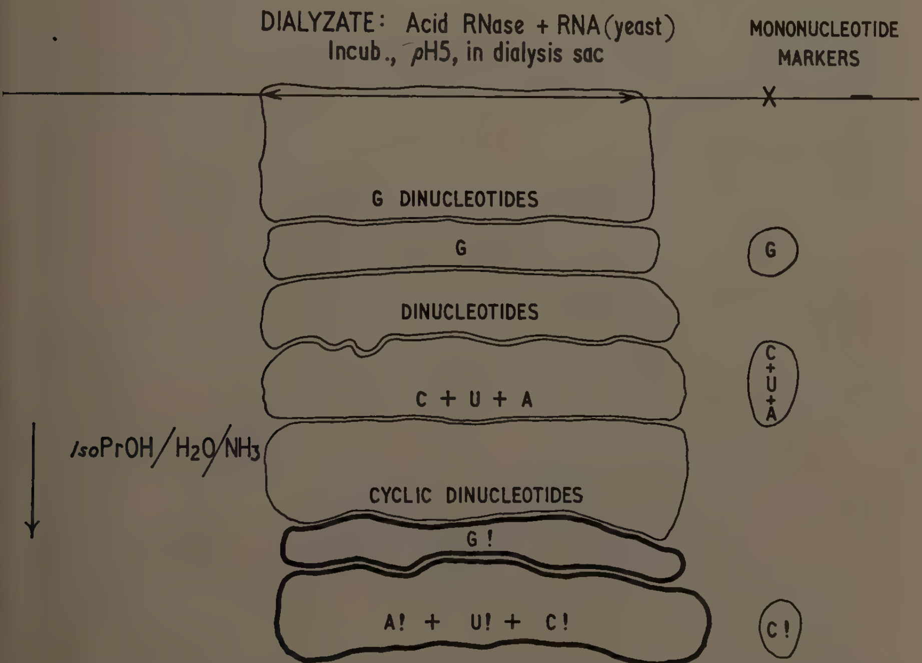

FIGURE 3. Tracing of paper chromatogram (examined under ultraviolet light), showing dialyzable products formed by action of acid RNase or RNA. Straight-chain adenylic, cytidylic, guanylic, and uridylic acids are denoted A, C, G, and U, respectively, and their cyclic derivatives (nucleoside 2′:3′-phosphates) A!, C!, G!, and U!, respectively.

Neither cyclic cytidylic acid nor cyclic adenylic acid is attacked by alkaline RNase as prepared by the several steps shown in FIGURE 2. There is appreciable attack on cyclic nucleotides (FIGURE 7) by the debris sedimented on centrifugation of the disrupted particles, but not further treated; however, this attack, which is more marked at *p*H 5 than at *p*H 8, is presumably due to contaminating acid RNase, being reduced by the heat treatment at *p*H 3.5 (FIGURE 7) and abolished by the final H_2SO_4 treatment.

The conclusion that alkaline RNase, in contrast with pancreatic RNase, does not attack cyclic nucleotides was confirmed by determining *p*H-activity curves (FIGURE 8) with an untreated mitochondrial fraction containing both acid and alkaline RNase. With cyclic adenylic acid, or with cyclic cytidylic acid (with

which the activity was much lower), there was a pH optimum in the acid region, as with RNA, but not in the alkaline region. Since cyclic cytidylic acid and cyclic adenylic acid differ somewhat in the pH optimum (FIGURE 8) and give the 3′ and the 2′ isomer, respectively, it is likely that 2 enzymes are involved—a conclusion supported by distribution data to follow.

The literature on specificities of RNases prepared from various sources and possibly varying in enzymic homogeneity shows no clear-cut analogies with the RNases we have studied. Kaplan and Heppel (1956) briefly describe a heat-stable liver RNase (alkaline) that can both form and attack cyclic pyrimi-

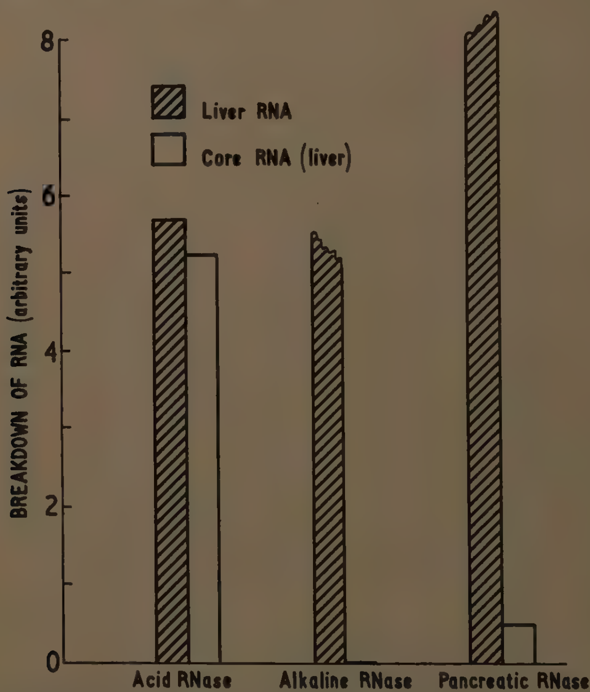

FIGURE 4. Comparison of RNA and of the "core" remaining after exhaustive digestion of RNA by pancreatic RNase as substrates for different RNase preparations.

dine nucleotides. Their preparation might contain a supernatant-fraction alkaline RNase differing in specificity from that in mitochondrial fractions, or it might contain the acid RNase that, as we have shown, attacks cyclic cytidylic acid; either possibility would be compatible with a preliminary observation we have made—that a supernatant fraction is active toward cyclic cytidylic acid even after heating.

Intracellular Distributions

Differential centrifugation of liver cytoplasm has been carried out in several experiments, the most elaborate of which is shown in FIGURE 9. The method of representation is that used by de Duve *et al.* (1955); for each of the 4 fractions, the product of the height of the block (relative specific activity) and the width

(percentage of cytoplasmic N in the fraction) is a measure of the amount of cytoplasmic activity found in the fraction.

Dealing first with the left half of FIGURE 9, representing experiments performed with the conventional 0.25 M sucrose medium, we have three marker enzymes as the basis for comparison, namely succinic dehydrogenase (mitochondria), acid phosphatase (lysosomes), and glucose-6-phosphatase (microsomes). The distribution of alkaline RNase is clearly bimodal, the activity being high

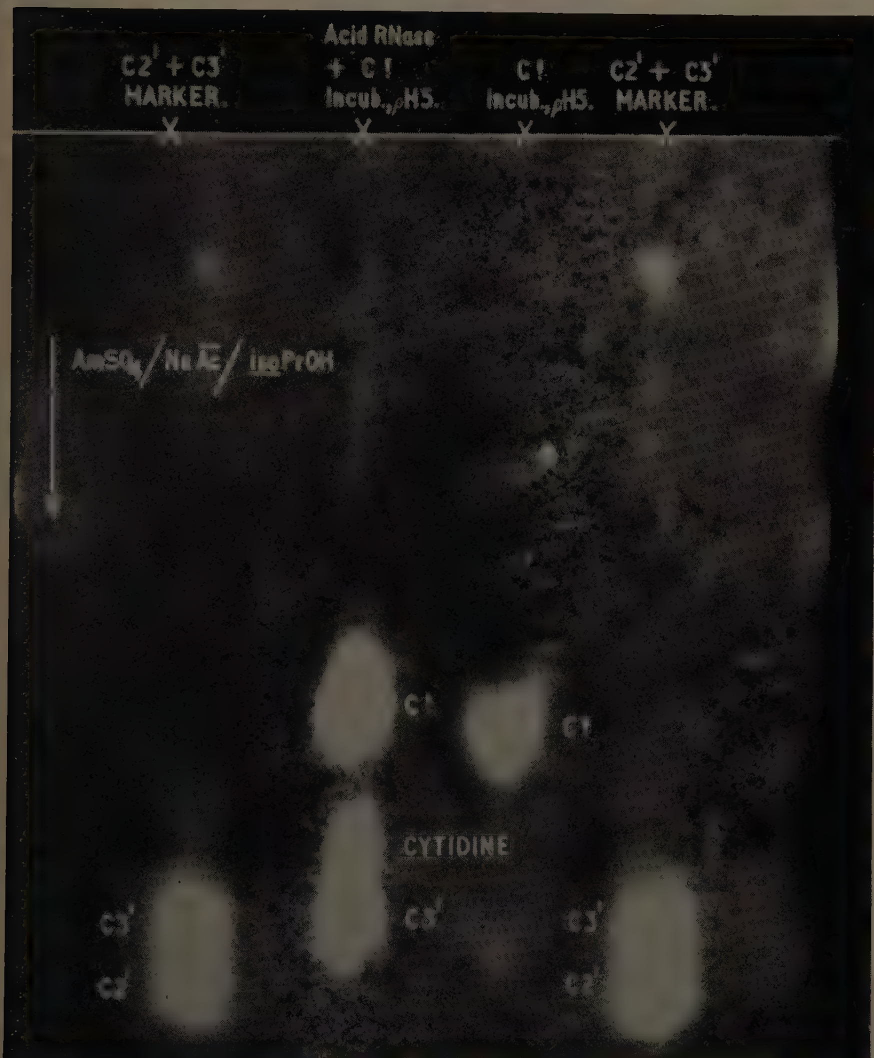

FIGURE 5. Photograph of chromatogram (under ultraviolet light), showing products obtained by action of acid RNase on cyclic cytidylic acid (C!). C2′ and C3′ denote the 2′- and 3′-isomers of cytidylic acid.

both in the second particulate fraction and in the supernatant fraction. Acid RNase has a distribution essentially similar to that of acid phosphatase if assayed with RNA or cyclic adenylic acid as substrate; this is in agreement

FIGURE 6. Photograph of 2-dimensional chromatogram (see text for procedure), showing products obtained by action of acid RNase on cyclic adenylic acid (A!). A2′ and A3′ denote the 2′- and 3′-isomers of adenylic acid.

with de Duve *et al.* (1955). With cyclic cytidylic acid as substrate, there is a notably high proportion of acid RNase in the supernatant fraction.

In a parallel series of experiments using the same centrifugation speeds (FIGURE 9, *right half*), we made use of a dextran-raffinose medium that had been devised by Birbeck and Reid (1956) to minimize damage to mitochondria.

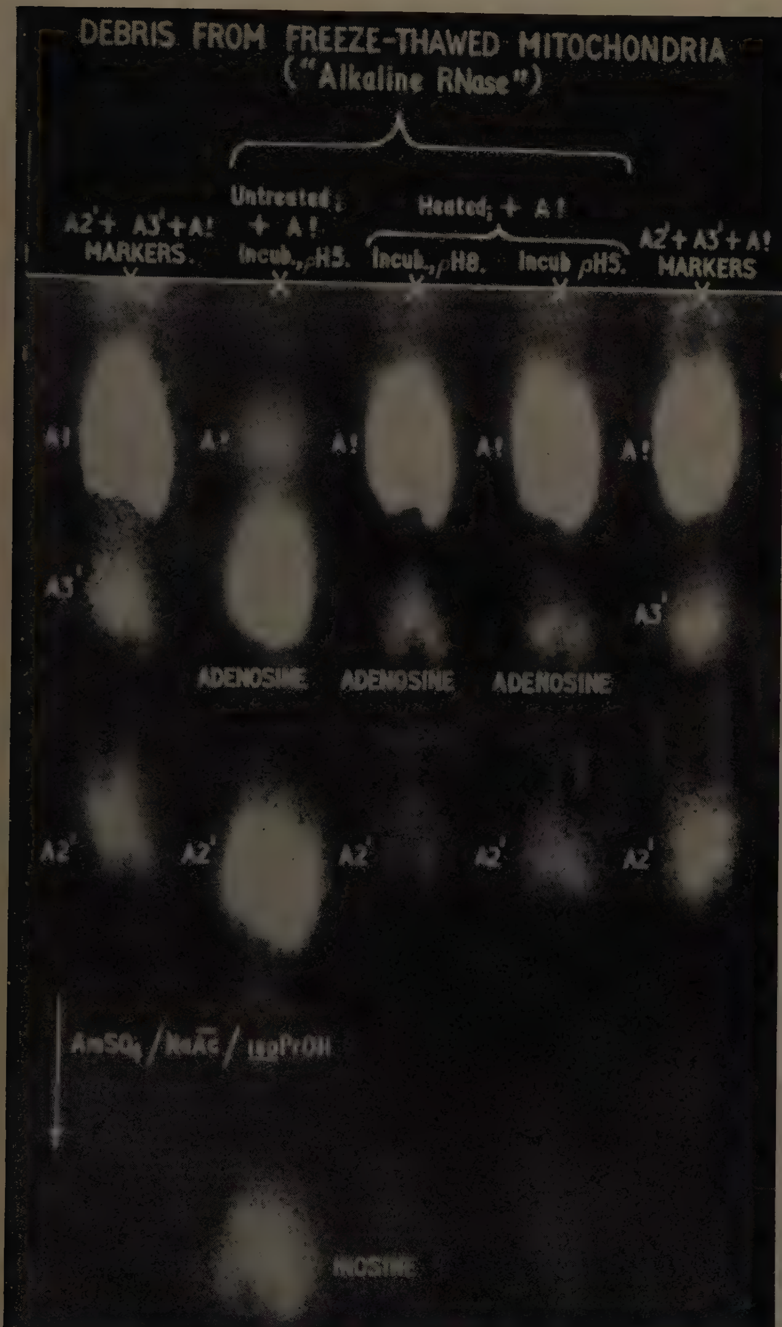

FIGURE 7. Photograph of chromatogram, showing diminution of attack on cyclic adenylic acid (A!) when the debris obtained from a freeze-thawed mitochondrial fraction is heated at pH 3.5 as in FIGURE 2. For A2′ and A3′ see FIGURE 6.

With this medium, mitochondria sediment as readily as in isotonic sucrose, but lysosomes and microsomes sediment less readily, as is evident from the apparent shifts in the distributions of acid phosphatase and glucose-6-phosphatase (FIGURE 9). Both alkaline RNase and acid RNase (whichever the

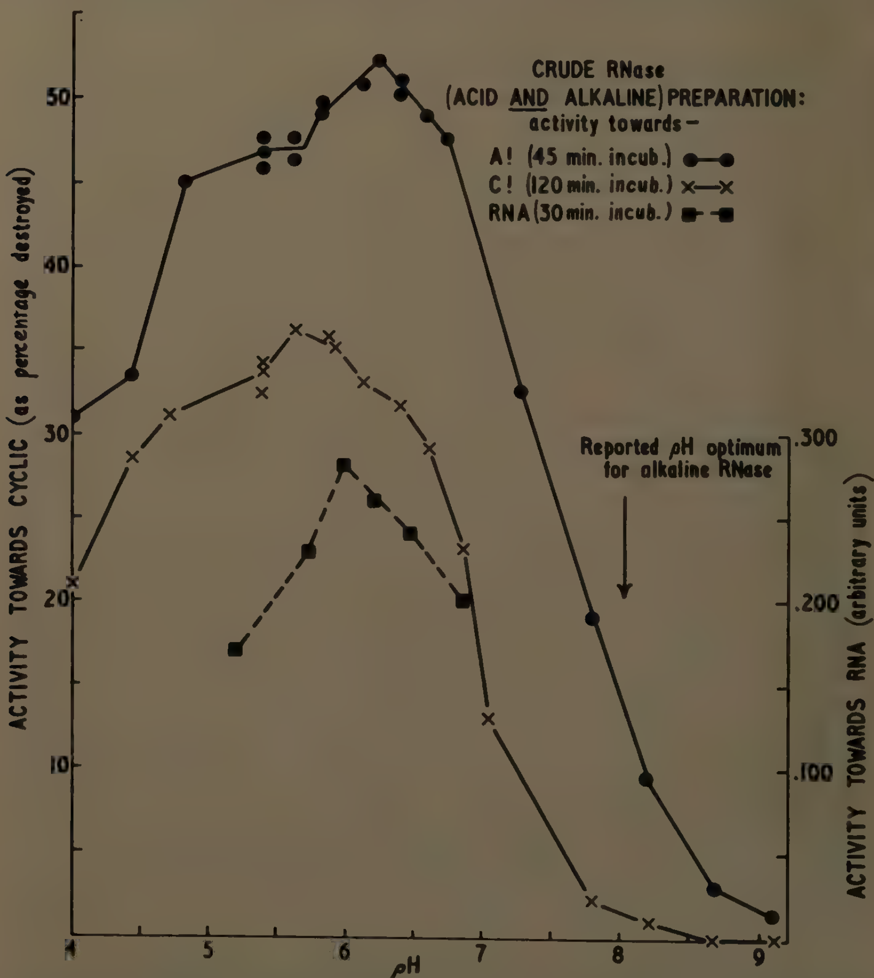

FIGURE 8. Activity, in relation to pH, for a crude mitochondrial fraction with different substrates, namely, cyclic adenylic (A!) or cytidylic (C!) acid, or RNA. Acetate-borate-cacodylate buffers were used.

substrate) show similar shifts, the concentration of activity (as of acid-phosphatase activity) now being particularly high in the second particulate fraction. The proportion of activity in the supernatant fraction is reduced for alkaline RNase, but not for acid RNase.

Our findings for RNases do not agree with those of de Lamirande and Allard (1957), whose results with a series of fractions isolated in hypertonic sucrose

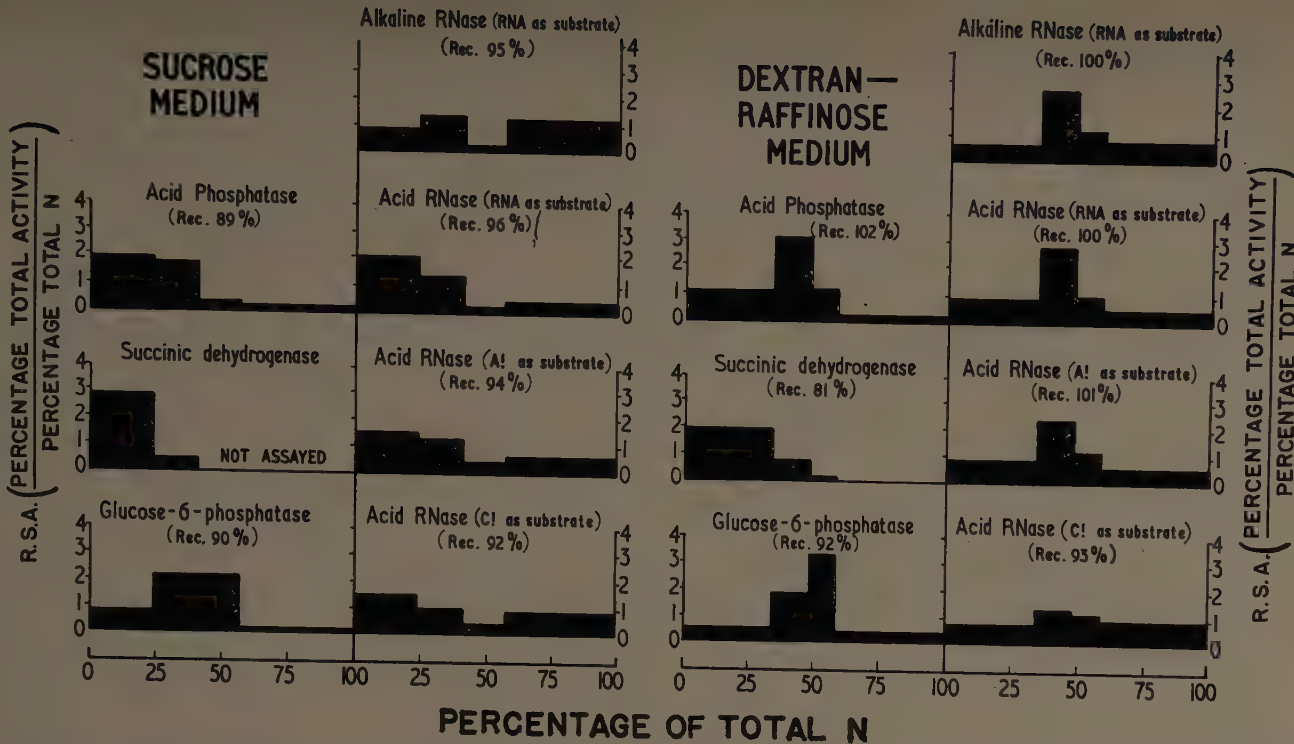

FIGURE 9. Enzyme distributions, among particulate and supernatant fractions from liver cytoplasm, plotted by method of de Duve *et al.* (1955) as explained in text. R.S.A. denotes relative specific activity and Rec. the recovery (in comparison with the original cytoplasmic fraction). See Birbeck and Reid (1956) for media, centrifugation conditions, and assay methods for the "marker" enzymes; for the 3 pellets, which were not washed, the values for gravity times minutes were 80,000, 600,000, and 8,700,000, respectively.

medium suggested that acid RNase is located in mitochondria, and alkaline RNase in both mitochondria and microsomes. With RNases, differences in assay procedures in different laboratories may well govern the results of such experiments. Our results for acid RNase distribution accord with those obtained under essentially similar conditions by de Duve *et al.* (1955).

Interpretation of distribution values. Taking into account the shifts in distribution observed when dextran-raffinose is used in place of sucrose, we conclude that both alkaline RNase and acid RNase (whichever the substrate) are present not in mitochondria, but in particles similar to if not identical with the lysosomes, which contain acid phosphatase. It is not proved, however, that RNases are located in, and uniquely in, lysosomes; with alkaline RNase, the activity, in comparison with that of acid phosphatase, is relatively low in the first (mitochondrial) fraction and high in the supernatant fraction. Assuming that there is indeed only one alkaline RNase in particles and that it is located in only one type of particle, the simplest hypothesis to explain our results is that alkaline RNase is present in particles that differ from lysosomes in being less readily sedimented.

Leakage of alkaline RNase from such particles during their isolation would account for the high activity in the supernatant fraction, this supposed leakage being reduced by the use of dextran-raffinose in place of sucrose. With catalase in sucrose medium, there is apparently such a leakage (from mitochondria), which can be prevented by addition of polyvinylpyrollidone or albumin to the medium (Greenfield and Price, 1956). Although alkaline RNase is still sedimentable when isolated particles are deliberately disrupted, any unintended disruption occurring with a crude homogenate might release the enzyme as a soluble complex with the inhibitor in the supernatant fraction. The high alkaline RNase activity of supernatant fractions could be explained by the alternative hypothesis that supernatant fractions contain an alkaline RNase different from that in mitochondrial fractions.

With acid RNase, close inspection of FIGURE 9 shows a slight excess in the first fraction (as compared with acid phosphatase), especially if RNA is the substrate; this is in agreement with de Duve *et al.* (1955). This would be understandable if acid RNase were present in particles distinguishable from lysosomes by being slightly more readily sedimented; but we hesitate to postulate yet another type of particle, and feel that lysosomes are probably the site.

The enzyme that attacks cyclic cytidylic acid has a diffuse distribution difficult to interpret; in contrast with alkaline RNase, the proportion in the supernatant is higher with dextran-raffinose than with sucrose. It is possible that the enzyme is a constituent of the cell sap that is adsorbed onto particles during their isolation; such adsorption phenomena are a well-known source of artifacts in distribution studies. An alternative possibility, compatible with a preliminary observation mentioned above, is that supernatant fractions contain an alkaline RNase that shows some activity toward cyclic cytidylic acid even at *p*H 5. Since in an experiment with liver thoroughly perfused *in situ* the usual high level of alkaline RNase was found in the supernatant fraction, it is unlikely that this activity is attributable to a blood RNase.

Changes in RNase Levels Under Various Conditions

Alkaline RNase will not be discussed here, its level in liver cytoplasm being essentially normal after hypophysectomy or adrenalectomy (Stevens and Reid, 1956) or in azo-dye carcinogenesis (de Lamirande and Allard, 1958; Reid and Lotz, 1958). In azo-dye carcinogenesis there is ample evidence, which need not be cited here, that acid RNase is likewise undiminished, in contrast with certain enzymes in the chain concerned with nucleotide catabolism; indeed, the acid RNase of the supernatant fraction may be increased.

Supernatant fraction–acid RNase activity, normally low in comparison with that in particles, increases markedly after hypophysectomy (especially if growth hormone is given) or adrenalectomy, but not after adrenodemedullation or treatment with thyroxine or thiouracil (Stevens and Reid, 1956; E. Reid, unpublished experiments). As suggested by de Duve, the acid RNase segregated in particles may be metabolically unavailable to the cell, whereas the small amount found free in the supernatant fraction may represent available enzyme.

It cannot be assumed that an increase in acid RNase signifies increased RNA catabolism. It has not been proved that acid RNase is a limiting enzyme, or even that it is a catabolic enzyme *in vivo*—although we feel justified in regarding it as catabolic in the absence of good evidence for an anabolic role. No consistent correlation between changes in RNA levels and changes in acid RNase levels has emerged from our studies of cell fractions in different endocrine states or in azo-dye carcinogenesis. When there is a rise in supernatant-fraction RNA, there is a marked rise in the acid RNase of the cytoplasm as a whole, if not of the supernatant fraction; but the converse does not hold. An increase in acid RNase can occur with no increase in RNA, not only in liver but also in kidney—an organ in which almost half of the cytoplasmic activity is found in the supernatant fraction (Reid and Stevens, 1958). Changes in acid RNase and RNA do not, then, show a correlation such as has recently been reported for their normal levels (in whole tissue) among different organs (Ledoux *et al.*, 1957).

With liver, we have studied the effect of adrenalectomy in some detail (Reid and Stevens, 1958). The rise in supernatant-fraction RNA, probably attributable to an increase in rate of synthesis, occurs earlier after operation than the rise in acid RNase (in contrast to the sequence of changes in DNA and DNase). The simplest interpretation of the changes after adrenalectomy is that when the level of supernatant-fraction RNA rises, its turnover becomes faster, the ultimate increase in acid RNase reflecting increased catabolism. We do not regard this interpretation as proved, and we feel it would be quite premature to assess what general role RNases play in the metabolism of RNA in the cell.

Summary

Alkaline RNase acts on RNA to give cyclic pyrimidine nucleotides that are not further attacked. Acid RNase furnishes both purine and pyrimidine cyclic nucleotides. Cyclic adenylic acid is further attacked to give 2′-adenylic acid, whereas cyclic cytidylic acid is attacked, probably by a different enzyme, to give 3′-cytidylic acid.

Alkaline RNase has an apparently bimodal distribution within the cell, the interpretation of which is discussed. Acid RNase assayed with RNA or cyclic adenylic acid as substrate may well be located in lysosomes. The enzyme that attacks cyclic cytidylic acid has a different distribution that is difficult to interpret.

Acid RNase may increase during azo-dye carcinogenesis or in certain endocrine states. The findings are discussed briefly in relation to the activity in supernatant fractions and to the levels of RNA in cell fractions.

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DISCUSSION

A. A. HAKIM (*Miami Heart Institute, Miami Beach, Fla.*): There is increasing evidence, from several reports, of the presence of different specific RNases

in certain biological materials. The heterogeneous nature of RNase in the urine is an interesting aspect of the problem of the fate of RNase.

Purified RNase preparations are obtained from human urine. The enzymes

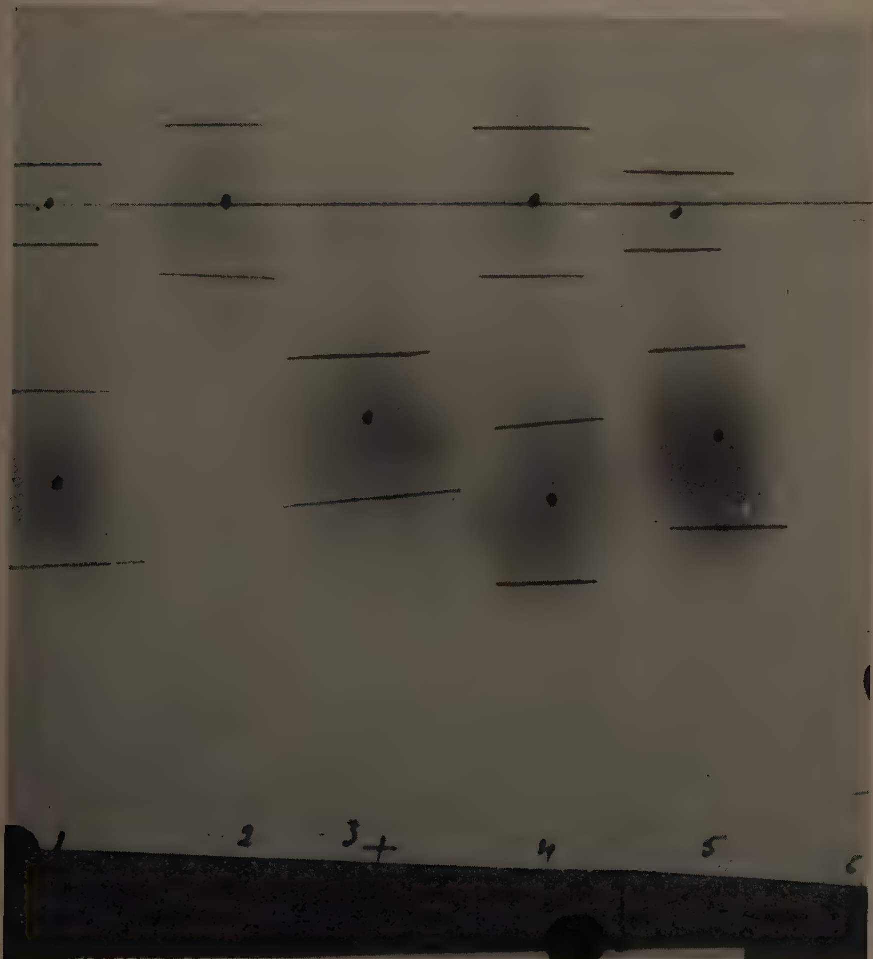

FIGURE 1. The electrophoretic mobility of urine RNases. The electrogram was run in 0.1 M acetate buffer, *p*H 5.6, with ionic strength of 0.05, at a potential difference of 300 v. and 0.5 mAmp. for 10 hours at 0° C. Column 1, free urine RNase; Column 2, combined urine RNase. Pancreatic crystalline RNase, urine acid RNase, and urine alkaline RNase are in columns 3, 4, and 5, respectively; column 6 contained external markers.

are prepared by published procedures to obtain the most purified samples of free and combined RNase, as well as of acid and alkaline RNases.

The electrophoretic mobilities of each of the urine enzymes as compared with pancreatic crystalline RNase are presented in FIGURE 1. Free urine RNase moved with the same electrophoretic mobility as crystalline pancreatic RNase;

TABLE 1
EFFECT OF HEAT ON RNASE ACTIVITIES

Preparations*	Percentages of original activity	
	pH 5.8	pH 7.8
Urine S_{11} acid RNase	40	92
Urine S_{11} alkaline RNase	92	96
Urine S_{21} acid RNase	50	65
Urine S_{21} alkaline RNase	10	64
Free urine RNase	35	55
Combined urine RNase	20	65

* All preparations were exposed to 98° to 100° C. for 10 min. at pH 7.2, then quickly cooled and activity determined.

TABLE 2
ACTION OF URINE RNASE ON SYNTHETIC SUBSTRATE

	Cytidine 2′,3′-phosphate		Uridine 2′,3′-phosphate	
	Extent of hydrolysis, percentages	Reaction products	Extent of hydrolysis, percentages,	Reaction products
Urine alkaline RNase	30	CMP-3′	22	UMP-3′
Urine acid RNase	80	CMP-3′	79	UMP-3′
Free urine RNase	92	CMP-3′(2′)	98	UMP-3′(2′)
Combined urine RNase	85	CMP-3′(2′)	82	UMP-3′(2′)

TABLE 3
ACTION OF URINE RNASE ON SYNTHETIC SUBSTRATE

	Adenosine 2′,3′-phosphate		Guanosine 2′,3′-phosphate	
	Extent of hydrolysis, percentages	Reaction products	Extent of hydrolysis, percentages	Reaction products
Urine alkaline RNase	12	AMP-3′	14	GMP-3′
Urine acid RNase	96	AMP-2′(3′)	97	GMP-2′(3′)
Free urine RNase	12	AMP-3′(2′)	25	CMP-3′(2′)
Combined urine RNase	35	AMP-3′(2′)	48	GMP-3′(2′)

TABLE 4
ACTION OF URINE RNASE ON YEAST RNA

	Mononucleotides liberated (moles per 100 moles)			
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid
Urine alkaline RNase	0	1.2	14.5	18.0
Urine acid RNase	16.0	11.2	13.5	14.0
Free urine RNase	8.5	10.5	14.8	16.5
Combined urine RNase	24.8	31.6	23.8	18.7

combined urine RNase remained at the line of application. Both urine acid and alkaline RNase consist of two enzymically active components: one, nonmobile remaining at the point of application; the second, mobile, with electromobility slightly different from that of pancreatic crystalline RNase.

RNase activity is determined by the rate of hydrolysis of uridine 2′,3′-phosphate, cytidine 2′,3′-phosphate, adenosine 2′,3′-phosphate, and guanosine 2′,3′-phosphate. The enzymic hydrolysis of yeast RNA by the above urine enzymes was determined.

The effect of heat on urine RNase activity is shown in TABLE 1. The enzymic activity was determined at *p*H 5.8 and *p*H 7.8. S_{11} is the urine sample collected on the eleventh day of the menstrual cycle; S_{21}, the sample collected the twenty-first day. Urine S_{11} alkaline RNase is thermostable, and urine S_{21} alkaline RNase was less stable; free RNase and combined RNase were thermolabile.

The specific enzymic activities of the urine enzymes on the synthetic substrates uridine 2′,3′-phosphate, cytidine 2′,3′-phosphate, adenosine 2′,3′-phosphate, and guanosine 2′,3′-phosphate are presented in TABLES 2 and 3.

Urine alkaline RNase acted slightly on the 4 cyclic mononucleotides (12 to 30 per cent hydrolysis). Urine acid RNase hydrolyzed the 4 cyclic mononucleotides to the extent of 80 to 97 per cent hydrolysis. Both urine acid and alkaline RNase produced the -3′ derivatives of cytidylic, uridylic, adenylic, and guanylic acid. Urine acid RNase produced 10 per cent of the mononucleotides as -2′ derivatives. Free urine RNase acted on the pyrimidine cyclic mononucleotides to the extent of 92 to 98 per cent hydrolysis, and hydrolyzed the purine cyclic mononucleotides slightly (12 to 25 per cent hydrolysis). In both cases, urine-free RNase gave 10 per cent of the mononucleotides as 2′ derivatives.

Combined urine RNase was activated by *p*-chloromercuribenzoate (1×10^{-5} M), and acted on pyrimidine cyclic mononucleotide (82 to 85 per cent hydrolysis) and on purine cyclic mononucleotides (35 to 42 per cent hydrolysis).

The action of the urine RNases on yeast RNA is presented in TABLE 4. Both free RNase and combined RNase produced the 4 mononucleotides from the nucleic acid. They produced less adenylic acid or guanylic acid than uridylic acid or cytidylic acid. Acid RNase produced the 4 mononucleotides from the nucleic acid, and larger quantities of adenylic and guanylic acids than those produced by free or combined enzymes. Urine alkaline RNase produced cytidylic acid and uridylic acid (14.5 and 18.0 moles per 100 moles, respectively). Differences in *p*H-activity curves and on ion-exchange resin also were observed and will be reported elsewhere.

Part III. Polynucleotide Phosphorylases

INTRODUCTORY REMARKS

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The availability of synthetic polyribonucleotides, particulary those with only one kind of nucleotide unit, has led to important studies of their macromolecular structure and the physicochemical properties of ribonucleic acid (RNA). Of particular importance was the finding of R. C. Warner in our laboratory that polyadenylic acid and polyuridylic acid interact in solution to form a stable aggregate. A brief description of his findings follows.

After preparation of various synthetic polynucleotides, while trying to determine whether the polymer made from mixtures of adenosine and uridine diphosphate (poly AU) was a true copolymer or consisted of separate chains of polyadenylic acid (poly A) and polyuridylic acid (poly U), Warner suggested that electrophoresis of the polymers should decide this question since, at a suitable *p*H, poly A and poly U should have different mobilities, and a mixture of these two polymers should be resolved into its components. Warner's electrophoretic studies showed that the mixture of poly A and poly U yielded, like poly AU, a single boundary with a mobility intermediate between that of poly A and poly U, suggesting an interaction of the two. Ultracentrifugal studies confirmed the fact that this interaction indeed resulted in the formation of an aggregate of increased molecular size. It was subsequently observed by Warner that the interaction between poly A and poly U led to a marked decrease in the absorption of light at wave length 260 mμ.

This work was followed by X-ray diffraction studies at Rich and his collaborators, from which they reached the important conclusion that the poly A + U aggregate has a double-stranded helical structure similar in many respects to that of DNA. Further experiments by Warner showed that the formation of the aggregate is due to hydrogen bonding between complementary adenine and uracil pairs. These results, which, in a way, provide direct experimental support for Watson and Crick's ideas on the structure of DNA, are likely to be of significance for a better understanding of the biological functions and the mechanism of molecular duplication of nucleic acids.

THE MECHANISM OF ACTION OF POLYNUCLEOTIDE PHOSPHORYLASE

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The purpose of this paper is to review certain aspects of the mechanism of action of polynucleotide phosphorylase and to present, rather briefly, some recent findings. The discussion will be concerned with studies carried out by S. Ochoa and his associates at New York University, New York, N. Y., and with work done at the National Institutes of Health. Reference will also be made to some recent work carried out in Bethesda by Grunberg-Manago. Some of the material to be presented has already been published, but a review may be profitable at this time. Certain of the unsolved problems that face investigators in this field also will be discussed.

Polynucleotide phosphorylase was discovered by Grunberg-Manago and Ochoa in extracts of *Azotobacter agile*.[1,2] Studies of the nature of nucleotide incorporation into nucleic acid in *Escherichia coli* led to a recognition of the same reaction by Littauer and Kornberg.[3,4,5] Beers[6] has made extensive studies of the enzyme from *Micrococcus lysodeikticus*, and some of this work will be presented in another paper in this symposium. Olmsted[7] has also reported studies dealing with polynucleotide phosphorylase from *M. lysodeikticus*.

The reaction catalyzed by the enzyme may be formulated as follows:

$$n \text{ nucleoside-}pp \xrightleftharpoons{Mg^{++}} (\text{nucleoside-P})^n + n \text{ P}_i \qquad (1)$$

where P_i is inorganic phosphate and nucleoside-*pp* represents a nucleoside 5′-diphosphate. Polymers are formed in this reaction that have all of the structural features of isolated ribonucleic acid (RNA) preparations and are attacked in a similar way by hydrolytic enzymes; the experimental evidence will not be reviewed here.

The reaction can be followed in various ways. In the forward direction, one can measure the release of inorganic phosphate or the formation of acid-insoluble polymer; in the reverse direction, one can assay the rate of phosphorolysis of polymers such as RNA or adenylate polynucleotide (poly A), or of suitable oligonucleotides. Finally, one can measure the P_i^{32} nucleoside diphosphate–exchange reaction. In this assay, adenosine diphosphate (ADP) or other nucleoside diphosphate is incubated with enzyme- and P^{32}-labeled inorganic phosphate, and the ratio of these components is such that no detectable net forward reaction occurs; the amount of radioactivity incorporated into ADP is then determined.

It is of interest to see how these different assays compare quantitatively when each is performed under optimum conditions. Table 1 shows data for an *E. coli* fraction (first ethanol step) prepared by Hilmoe according to Littauer and Kornberg,[5] and for a fraction from *A. agile*[8] supplied by S. Mii and S. Ochoa.

The results for all of these assays are given in the same units—that is, μmoles

per hour per milligram of protein. It is evident that measurement of P_i release in the forward reaction (EQUATION 1) gives much higher values for specific activity of the enzyme fractions than is true for the other assays. Some possible explanations for these differences will become obvious as each of the three activities of the enzyme is discussed in turn.

The forward reaction, which will be considered first, is most commonly followed by measurement of the rate of P_i formation from a single nucleoside diphosphate, or from a mixture of nucleoside diphosphates. With the enzyme preparation from *E. coli* or the earlier fractions obtained during purification of the *Azotobacter* enzyme, polymerization occurs at a linear rate until equilibrium is approached. Further, there is no stimulation, or priming, by the addition of polymers or oligonucleotides (this is in contrast to results with highly purified *Azotobacter* fraction, described below). It should be emphasized, however, that oligonucleotides of suitable structure may participate in the polymerization reaction even in cases where they afford no stimulation of enzyme activity; they are, in fact, incorporated into the polymer that is formed.

TABLE 1
COMPARISON OF THREE ASSAYS FOR POLYNUCLEOTIDE PHOSPHORYLASE

	Micromoles per hour per milligram of protein	
	E. coli	*A. agile*
P_i formation from ADP*	280	1000
Phosphorolysis of poly A†	20	45
ADP-P_i^{32} exchange‡	35	100

* Assay described in Mii and Ochoa.[8]
† Assay described in Singer.[12]
‡ Assay described in Grunberg-Manago *et al.*[2]

An example of the type of oligonucleotide incorporated is the trinucleotide, pApApA.* This compound contains a phosphomonoester group at carbon 5′ of the first adenosine residue, and the terminal nucleoside contains an unsubstituted hydroxyl group at carbon 3′. Polynucleotide phosphorylase catalyzes the addition of a mononucleotide unit to the terminal nucleoside of pApApA, and this process continues, giving a polymer chain with pApApA forming its beginning portion.

A considerable advance in our understanding of polynucleotide phosphorylase came when Mii and Ochoa[8] discovered a lag phase in the polymerization of ADP, inosine diphosphate (IDP), uridine diphosphate (UDP), and cytidine diphosphate (CDP), which was overcome by addition of RNA or of certain of the biosynthetic polymers. This lag period was found only with highly purified *A. agile* fractions. Singer *et al.*[9] found that oligonucleotides such as pApApA also overcame the lag period.† In addition to stimulating the reaction in this

* This and other abbreviations used follow the system described in the "Instructions to Authors" in *The Journal of Biological Chemistry*, September, 1958.

† These studies were carried out with the *A. agile* fractions provided by Ochoa. Recently, the essential findings were confirmed with a fraction purified from *A. agile* by Singer and Hilmoe. However, the lag period was not as striking as in the earlier work and further purification of their fraction is indicated.

manner, they were incorporated into the polymer by the same mechanism outlined above for more crude *Azotobacter* fractions.

These studies were extended to include oligonucleotides in which the hydroxyl group at C3′ of the terminal nucleoside residue was blocked by a phosphomonoester residue. An example is ApApUp. With this compound (and its homologues) esterification to add new mononucleotide units and, thereby, to lengthen the chain is impossible. Consequently, oligonucleotides of this type are not incorporated, yet the surprising observation was made that they stimulate the

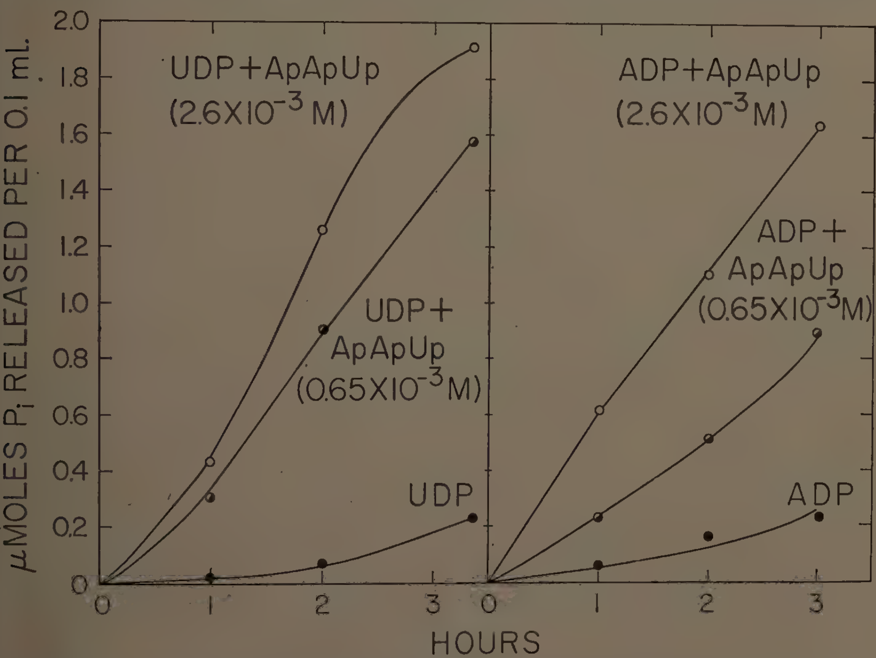

FIGURE 1. The effect of ApApUp on the lag in the polymerization of ADP and UDP with purified *A. agile* polynucleotide phosphorylase. The reaction mixtures contained Tris buffer (*p*H 8.2), 7.5 μmoles; ethylenediamine tetraacetate, 0.02 μmole; ADP or UDP, 3 μmoles in a total volume of 0.05 ml. In the ADP experiments the reaction mixtures also contained 0.5 μmole $MgCl_2$ and 5.1 × 10^{-4} mg. enzyme (specific activity 150 by the "exchange" assay); in the UDP experiments they contained 1.5 μmoles $MgCl_2$ and 1.1 × 10^{-3} mg. enzyme. The concentrations of oligonucleotides are indicated on the figure. The reaction was followed by determination of the release of P_i. Incubation temperature, 37° C.

polymerization reaction, overcoming the lag period both for ADP and UDP (FIGURE 1).

There are no data to explain how these nonincorporated oligonucleotides act in overcoming the lag period. It is possible that such compounds and the various polymers stimulate the reaction in a similar fashion. However, there is no apparent specificity to be observed with oligonucleotides, while specificity relationships have been found with polymers.[8] The great interest in primers that are not incorporated into new chains lies in the possibility that large polynucleotide molecules of this kind may have a directing influence on the composition of the polymer synthesized.

The behavior of guanosine diphosphate (GDP) is unique because, when present alone, it cannot be polymerized by any available preparation of polynucleotide phosphorylase, whether from *E. coli* or *A. agile*. There is no reaction with a large excess of enzyme or after many hours of incubation, even with those fractions that show no lag period with other nucleoside diphosphates. With the same enzyme preparations, GDP is well utilized if mixed with other nucleoside diphosphates; thus, Grunberg-Manago *et al.*[2] described the preparation of poly AGUC several years ago.

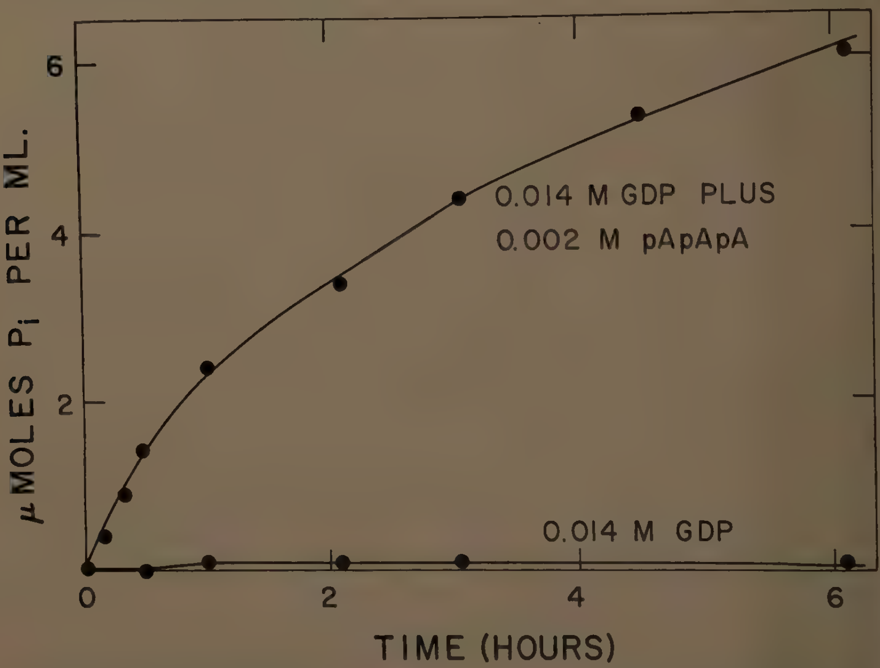

FIGURE 2. The polymerization of GDP in the presence of pApApA. The reaction mixtures contained Tris buffer (*pH* 8.2), 23 μmoles; $MgCl_2$, 1.5 μmoles; ethylenediamine tetraacetate, 0.06 μmole; GDP, 2.1 μmoles; and *Azotobacter* polynucleotide phosphorylase, 0.012 mg., in a final volume of 0.15 ml. The experiment shown in the upper curve included, in addition, 0.3 μmole pApApA. The reaction was followed by determining the release of inorganic phosphate. Incubation temperature, 37° C.

If an oligonucleotide with a free C-3′ hydroxyl group is included in the incubation mixture, a polymerization reaction involving GDP does take place (FIGURE 2). An example of such an oligonucleotide is pApApA, but others would also serve. The hydroxyl group is esterified in the enzymatic reaction, forming a phosphodiester bond and adding the first guanosine monophosphate residue:

$$\text{pApApA} + \text{GDP} \rightleftarrows \text{pApApApG} + P_i \qquad (2)$$

The tetranucleotide, pApApApG, is the first major product of the reaction; it has been separated by paper chromatography followed by rechromatography

in another solvent system. Hydrolysis in 1N HCl yielded adenine and guanine in a ratio of 3.2:1.0, the theoretical being 3.0:1.0. Digestion by alkali gave the expected products, adenosine 3′,5′-diphosphate, adenosine 3′-phosphate, the corresponding 2′-isomers, and guanosine. Partial hydrolysis with snake venom phosphodiesterase (see Hilmoe, this monograph) gave the expected products.

FIGURE 3 illustrates the time course of the reaction. The primer, pApApA, disappears as it is incorporated. The concentration of the compound just discussed, pApApApG, first rises and then falls as the addition of guanosine

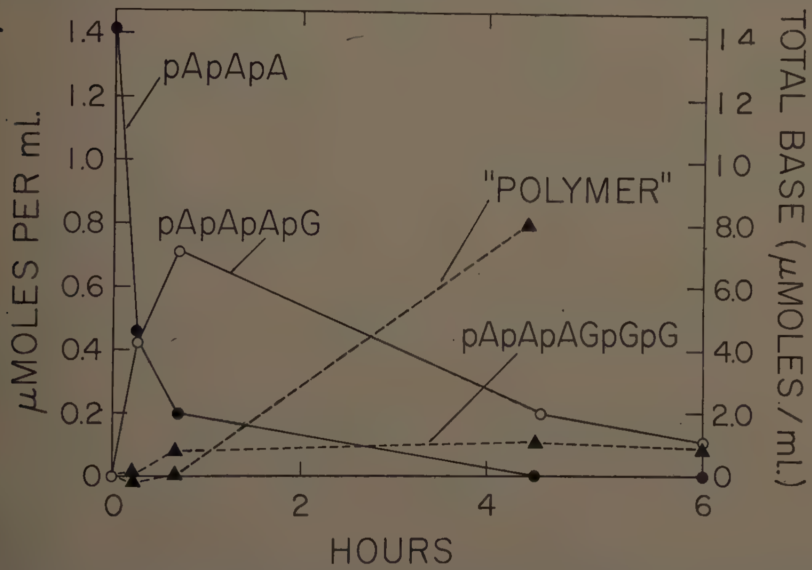

FIGURE 3. The polymerization of GDP in the presence of pApApA. The incubation mixture contained 0.08 mg./ml. of *Azotobacter* enzyme plus the following, in micromoles per milliliter: Tris buffer (*p*H 8.2), 150; $MgCl_2$, 10; ethylenediamine tetraacetate, 0.4; GDP, 13.7, and pApApA, 2. Aliquots were removed at different time intervals for quantitative paper chromatography. No polymer could be detected at 20 and 40 min; it appeared after 60 min. and increased progressively to reach a value equivalent to 8 µmoles of base per milliliter after 6½ hours. Temperature, 37° C.

monophosphate residues takes place, to give larger oligonucleotides and, finally, polymer. The polymer is nondialyzable against 0.001 M ethylenediamine tetraacetate, is precipitated by 2.5 per cent perchloric acid or 2 volumes of ethanol, and it does not migrate on paper chromatograms. Unfortunately, the amount of primer required for a reasonably rapid reaction with GDP is rather large. Thus, with 0.004 M pApApA the rate of polymerization of GDP is one fourth as fast as with ADP, and with less primer it falls off sharply. With limited amounts of enzyme it then becomes difficult to add more than about 9 guanosine monophosphate residues, on the average, per unit of primer and to do this on a reasonable scale.

In contrast to the situation with ADP and UDP, oligonucleotides such as ApApUp do not stimulate polymerization of GDP. Polymers are also inac-

tive, either because of inhibitory interactions or, perhaps, because the available concentration of terminal nucleoside residues onto which a guanylic acid residue can add is too low.

In the reverse reaction catalyzed by this enzyme, namely, the phosphorolysis of polynucleotides to form nucleoside diphosphates, poly A and poly U are rapidly attacked by polynucleotide phosphorylase.[2, 5] Substantial rates of phosphorolysis have been found for tobacco mosaic virus RNA, turnip yellow mosaic virus RNA, and highly polymerized yeast RNA.[10] On the other hand, commercial yeast RNA, which has been treated with alkali, is phosphorolyzed very slowly.[5]

Commercial yeast RNA is considered to be made up of relatively short chains, many of them terminated by 3′-phosphomonoester and 2′,3′-cyclic phosphoryl end groups.[11] One possible explanation for its poor rate of phosphorolysis would be the resistance offered by such end groups. According to Singer,[12] oligonucleotides as large as a pentanucleotide are not phosphorolyzed if they possess the types of end group just mentioned. By contrast, oligonucleotides with a 5′-phosphomonoester end group are rapidly attacked until the molecule is reduced in size to a compound with 2 nucleoside residues. Conceivably, then, commercial RNA is resistant to enzymatic splitting because most of the chains have an unfavorable end-group structure. However, Grunberg-Manago has found that phosphorolysis of commercial RNA proceeds to the extent of at least 90 per cent.[13] Possibly in a molecule larger than a pentanucleotide, enzymatic break down is possible even with a 3′-phosphomonoester or 2′,3′-cyclic phosphoryl end group.

In a preparation of RNA consisting of chains with an unsubstituted terminal nucleoside residue mixed with other chains in which a phosphate group is monoesterified at C-3′ of the terminal residue, would chains of the second type inhibit the rate of phosphorolysis of chains that did not have such terminal phosphate groups? With this question in mind, Singer *et al.*[14] recently measured the rate of phosphorolysis of "5′-ended" oligonucleotides in the presence of "3′-ended" oligonucleotides. The results are shown in TABLE 2. It is apparent that ApApUp inhibits the rate of phosphorolysis of pApApA, but the effect is not great, even though a substantial amount of the "3′-ended" oligonucleotide is present. Very little information is available on the end-group structure of RNA preparations, but the possibility should be kept in mind that inhibitory effects of this kind may be operating.

A second reason for a slow rate of phosphorolysis of polynucleotides is the formation of multi-stranded chains. The interaction of poly A and poly U was first observed by Warner,[15] and various aspects of this subject are discussed elsewhere in this monograph. Ochoa observed that when poly A and poly U were mixed in the ratio of 1:1 the rate of phosphorolysis was considerably depressed, as compared with the rate for either polymer by itself.[10] Grunberg-Manago made a similar observation and also noted that phosphorolysis was suppressed almost completely with a ratio of poly A to poly U of 1:2; under these conditions, a triple-stranded chain is formed.[16] Her results with poly I are also of interest. This polymer is known, from the work of Rich,[17] to exist as a random coil in dilute salt solution and as a triple helix in 0.6 M KCl. It was noted that phosphorolysis of poly I proceeded readily in dilute salt

and was suppressed nearly completely in 0.6 M KCl, whereas poly A was equally reactive in both concentrations of salt.

At this point it is profitable to discuss a third reaction catalyzed by polynucleotide phosphorylase, namely the exchange of P_i^{32} and nucleoside diphosphate. The exact mechanism of this interesting reaction is unproved. An explanation favored by Ochoa is that the incorporation of P_i^{32} into nucleoside diphosphate results from synthesis of a small amount of polynucleotide, followed by its phosphorolysis. This is a reasonable supposition and suggests the possibility that oligonucleotides might stimulate the rate of the exchange reaction.

Recently, Singer *et al.*[14] studied the effect of pApA and pApApA on the rate of exchange of inorganic P^{32} with ADP and UDP; a significant stimulation was

Table 2

Phosphorolysis of Oligoribonucleotides*

Substrate†	Addition†	Rate of phosphorolysis‡
pApApA (1.3)		18.1
pApApApA (0.8)		55.7
poly A (0.9)		7.2
pApApA (1.3)	ApApUp (1.1)	14.5
pApApApA (0.8)	ApApUp (1.1)	55.7
Poly A (0.9)	ApApUp (1.1)	6.6

* The reaction mixture contained, in 0.125 ml.: Tris buffer (*p*H 8.2), 5 μmoles; $MgCl_2$, 0.5 μmole; P_i^{32}, 3.05 μmoles containing 448,000 cpm (polynucleotides as indicated), and 0.002 mg. *E. coli* polynucleotide phosphorylase (first ethanol step[5]). After 1 hour the reactions were stopped with perchloric acid, the nucleotides adsorbed onto charcoal, and the charcoal washed free of P_i^{32} and suspended in ethanolic NH_3. Aliquots of the suspension were plated and counted.

† The numbers in parentheses are micromoles of polynucleotide per milliliter; for poly A this is expressed as adenine residues.

‡ Rates of phosphorolysis are expressed as micromoles of P_i^{32} incorporated into nucleotides per hour per milligram of enzyme.

obtained (TABLE 3). These results agree with similar data obtained by Mii and Ochoa.[18] The experiments shown in TABLE 3 were carried out with a preparation of *Azotobacter* enzyme provided by Ochoa, which catalyzes the polymerization of ADP and UDP only after a lag period. This lag period is overcome by concentrations of pApA and pApApA similar to those described here. The maximum stimulation observed was not large, amounting to 140 per cent.

It was hoped that more striking results might be obtained with GDP, since its polymerization shows an absolute requirement for an oligonucleotide primer. By suitable adjustment of the concentration of $MgCl_2$ and the GDP-P_i ratio it was possible to obtain a rate of P_i^{32}-GDP exchange comparable to exchange rates for ADP and UDP* (TABLE 4). Under these conditions the rate of P_i^{32}-GDP exchange was stimulated more than threefold by 5×10^{-3} M pApApA. A control experiment with pApApA, but no GDP, showed a very small incorporation of P_i^{32} into nucleotide material; a correction for this was applied.

* A detailed account of this is to be published.

To recapitulate: the enzyme preparations catalyze the polymerization of GDP only in the presence of an oligonucleotide whose configuration allows the addition of guanosine monophosphate units. Such oligonucleotides also stimulate the P_i^{32}-GDP exchange reaction. This effect is understandable if one visualizes the rapid and reversible addition of nucleotide units to the added

TABLE 3

EFFECT OF OLIGONUCLEOTIDES ON THE ADP-P_i^{32} AND UDP-P_i^{32} EXCHANGE REACTIONS*

Nucleotide added†	Micromoles P_i^{32} incorporated into charcoal-adsorbable nucleotides, per hour, per milligram	Stimulation per cent
ADP	71	
ADP and pApA, 3×10^{-3} M	131	85
ADP and pApApA, 5×10^{-4} M	176	140
UDP	37	
UDP and pApA, 3×10^{-3} M	51	38
UDP and pApApA, 5×10^{-4} M	127	135
pApApA	6	

* The reaction mixtures contained 10 μmoles Tris buffer (*p*H 8.2), 0.02 μmole ethylenediamine tetraacetate, 0.5 μmole $MgCl_2$, 0.8 μmole inorganic P^{32} (92,000 cpm/μmole) in a total volume of 0.1 ml. In the ADP experiment, 0.63 μg. and, in the UDP experiment, 1.25 μg. of *A. agile* polynucleotide phosphorylase were added.

† Nucleotides were added in the following amounts: ADP, 0.5 μmole; and UDP, 1.0 μmole. Incubation was at 37° C. for 30 min.

TABLE 4

COMPARISON OF EXCHANGE RATES WITH GDP, ADP, AND UDP*

Substrate	Micromoles P_i^{32} incorporated into charcoal-adsorbable nucleotides, per hour per milligram protein
GDP	45
ADP	51
UDP	49

* Reaction mixtures (0.1 ml.) contained 10 μmoles Tris buffer, (*p*H 8.2), 0.02 μmole ethylenediamine tetraacetate, 2.6 μg. *A. agile* polynucleotide phosphorylase, 0.8 μmole P_i^{32} (specific activity, 60,000 cpm/μmole), and one of the following combinations: 0.4 μmole GDP and 1 μmole $MgCl_2$, 0.5 μmole ADP and 0.5 μmole $MgCl_2$, or 1 μmole UDP and 0.5 μmole $MgCl_2$. After incubation for 1 hour at 37° C. the labeled nucleoside diphosphate produced in the exchange reaction was separated from inorganic P^{32} by adsorption onto charcoal and its radioactivity measured.

primer. More difficult to comprehend is the fact that a stimulation of 220 per cent was obtained with ApUp, a dinucleotide containing a 3′-phosphomonoester end group. This compound is not incorporated and does not support synthesis of poly G. This is the second example cited in this paper of stimulation of polynucleotide phosphorylase by ApUp and ApApUp. In each case, the possible mechanism is an intriguing problem for future study.

Brief reference may be made to an interesting effect of polymers on the exchange reaction. Mii and Ochoa[8] reported that the lag in the rate of polymerization of ADP and UDP found with purified *Azotobacter* enzyme can be

overcome by poly A and poly U, respectively. However, if the opposite pairs are used (for example, ADP and poly U), then the polymerization reaction is actually inhibited. Recently, Singer *et al.*[14] observed somewhat similar effects in studying the exchange reaction. Thus, when poly A, ADP, and P_i^{32} are present in a single reaction mixture, the incorporation of P_i^{32} happens to be equal to the sum of the ADP-P_i^{32} exchange and the phosphorolysis of poly A, as measured separately. Similar data were obtained for the combination of UDP, poly U, and P_i; however, when poly A is added to UDP or poly U to ADP, both exchange and phosphorolysis are inhibited. No such inhibition is

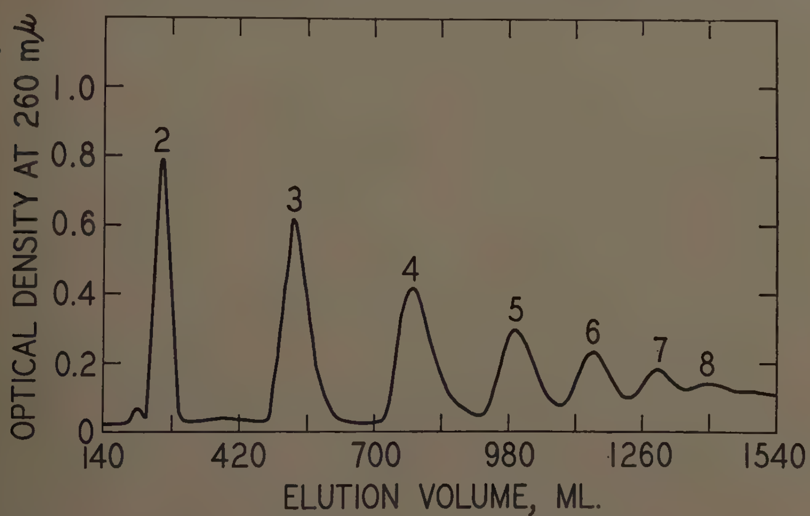

FIGURE 4. Separation of oligoribonucleotides on an "Ecteola" column. Poly AU was made by incubating equimolar quantities of ADP and UDP with polynucleotide phosphorylase; 30 mg. of the polymer was then digested with pancreatic ribonuclease and placed on a 44- × 1-cm. column of the modified cellulose adsorbent, Ecteola. Gradient elution was used, with 750 ml. of 0.015 M lithium acetate (*p*H 5.5) in the mixing chamber and 750 ml. of 0.4 M lithium chloride-0.015 M lithium acetate in the reservoir. Flow rate was 1.3 ml./min. The peaks represent a homologous series of oligonucleotides; thus, Peak 2 is ApUp, Peak 3 is ApApUp, Peak 4 is ApApApUp, and so on.

observed with GDP in the presence of either poly A or poly U. This is also true for thymine riboside pyrophosphate.

Brief reference should be made to methods used in the separation of the several homologous series of oligonucleotides, which have been so useful in studies of polynucleotide phosphorylase. In the past, they have been isolated by paper chromatography[12] and on Dowex 1-2x columns.[19] Recently, in Khorana's laboratory, good separations of these oligoribonucleotides were achieved by Tener and Heppel (unpublished data) using modified "Ecteola" columns.[20] Eluting conditions were similar to those previously employed* for oligodesoxyribonucleotides. FIGURE 4 shows the elution diagram obtained with the homologous series beginning ApUp, ApApUp. A similar record was obtained with the series beginning pApA, pApApA.

* See discussion in Tener *et al.*, this monograph.

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AN EXAMINATION OF THE MECHANISM OF ACTION OF POLYNUCLEOTIDE PHOSPHORYLASE BY THE USE OF ACRIDINE ORANGE*

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Introduction

The reactions of acridine orange with nucleic acids have been shown to be of two types.[2] One reaction produces a red complex, a shift of the dye spectrum toward the violet, and a decrease of the green fluorescence. This reaction is attributed to ribonucleic acid (RNA). The other reaction produces a marked intensification of the green fluorescence of the dye without any significant spectral shift. This green complex is attributed to desoxyribonucleic acid (DNA). Numerous studies based on these two reactions[3-5] have been carried out on the histochemical identification and distribution of RNA and DNA.

Recently, however, Beers *et al.*[6] have shown that the specificity of the reaction is determined by the relative distribution of the dye molecules along the chain of the polynucleotide (the red complex I) and at the end of the chain (the green complex II). When the ratio of RNA to dye is sufficiently large, complex II is formed at the expense of complex I. DNA in a native state forms only complex II, regardless of concentration, but when denatured by heat or alkali, forms complex I at low nucleotide-dye ratios and low ionic strength.[7]

Because complex II appears to represent the reaction of a terminal group of polynucleotides with the dye, acridine orange should be a valuable analytical reagent for determining the population of polymer molecules, the number-average molecular weight, the number-average degree of polymerization and, if the weight-average molecular weight is known, the degree of polydispersity of the polymer preparation. Of particular importance is the relationship between the population of molecules and the enzymatic processes leading to their formation or break down. The distinction between random and terminal group splitting of the polymer can be made. This paper presents some of the initial findings observed in the polymerization of polyadenylic acid (poly A) with polynucleotide phosphorylase (polyase) obtained from *M. lysodeikticus*. Attention has been focused on the role of primers in the polymerization process.

Materials and Methods

The polyase preparations were obtained from acetone-dried cells of *M. lysodeikticus*, according to procedures published elsewhere.[8, 9] Polymerization reactions were followed by measuring the rate of inorganic phosphate released.[9] The polymer was isolated by precipitation from the reaction mixture with 5 per cent $HClO_4$ or by salting out with KCl. Poly A used in the titration studies with acridine orange was deproteinated by the method of Sevag.[10] Concen-

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trations of the polymer and of nucleoside diphosphate substrates were estimated from the organic phosphate content.*

Before use, the acridine orange† was recrystallized twice from methanol. A major portion of the work was completed before it was discovered that the dye is not prepared as the hydrochloride salt, as described by the distributing agencies,[11] but as the $ZnCl_2$ salt. Positive identification of the Zn was made from the characteristic diphenylthiocarbazone reaction at *p*H 6 in CCl_4. Preliminary studies indicate that the metal is present in equimolar proportions with the dye. A Zn-free preparation was made by adsorbing the dye from an aqueous solution on an Amberlite IR-120(H) column, eluting the Zn with 1.0 N HCl and the dye with 8.0 N HCl or HNO_3. A generous sample of chromatographically pure dye has been received from Adrien Albert.

Titration of the polymer with the dye was performed with a microsyringe. To 3 ml. of a buffered solution of the polymer [0.01 M tris(hydroxymethyl) aminomethane, *p*H 8.0] in a Beckman cuvette of 1-cm. path length are added aliquots of an aqueous solution of the dye. The Zn salt is readily soluble in water, but the free base must be converted to acid form before sufficient quantity can be dissolved. The optical density of the polymer-dye mixture is read at 502 mμ, the peak of absorption for complex II. Approximately 10 to 15 min. are allowed between the additions of the dye aliquots (2×10^{-3} ml.) to assure completion of the complex II reaction.

Results

Titration studies. FIGURE 1 shows a typical series of titration curves with different concentrations of poly A. The initial slope at low dye concentration is equal to the extinction coefficient of complex II. The absorption spectrum of the dye in this region is identical with that of complex II (Figure 1 in Beers *et al.*[6]). The limiting slopes at high dye concentration are the same for each polymer concentration and are equal to the extinction coefficient of complex I. The difference spectrum, obtained by subtracting the optical densities at low dye concentration from the optical densities at high dye concentration along the linear portion of this slope, is identical with the absorption spectrum of complex I (Figure 1 in Beers *et al.*[6]).

The intermediate nonlinear region represents the formation of a mixture of complex I and complex II. An analysis of the spectral changes accompanying the transition from complex II to complex I formation shows no intermediate third species, except in the presence of high salt concentration. Therefore, the intercept of the extrapolated linear slopes represents (1) the optical density increment resulting from the formation of complex II, and (2) the amount of dye required to saturate the complex II sites. These are plotted in FIGURE 2 as functions of the polymer concentration. This dye titration method is extremely sensitive for measuring complex II, detecting concentrations of 5×10^{-7} moles or less, or, in a 1-ml. sample, 5×10^{-10} moles. In the

* Adenosine diphosphate (ADP) was obtained from either Pabst Brewing Co., Milwaukee, Wis., or Sigma Chemical Company, St. Louis, Mo.

† Manufactured by the National Aniline Division of the Allied Chemical and Dye Corporation, New York, N.Y.

usual polymer preparations this represents less than 1 complex II site per 200 to 300 nucleotide bases.

Varying the ionic strength alters the quantitative relationship between the

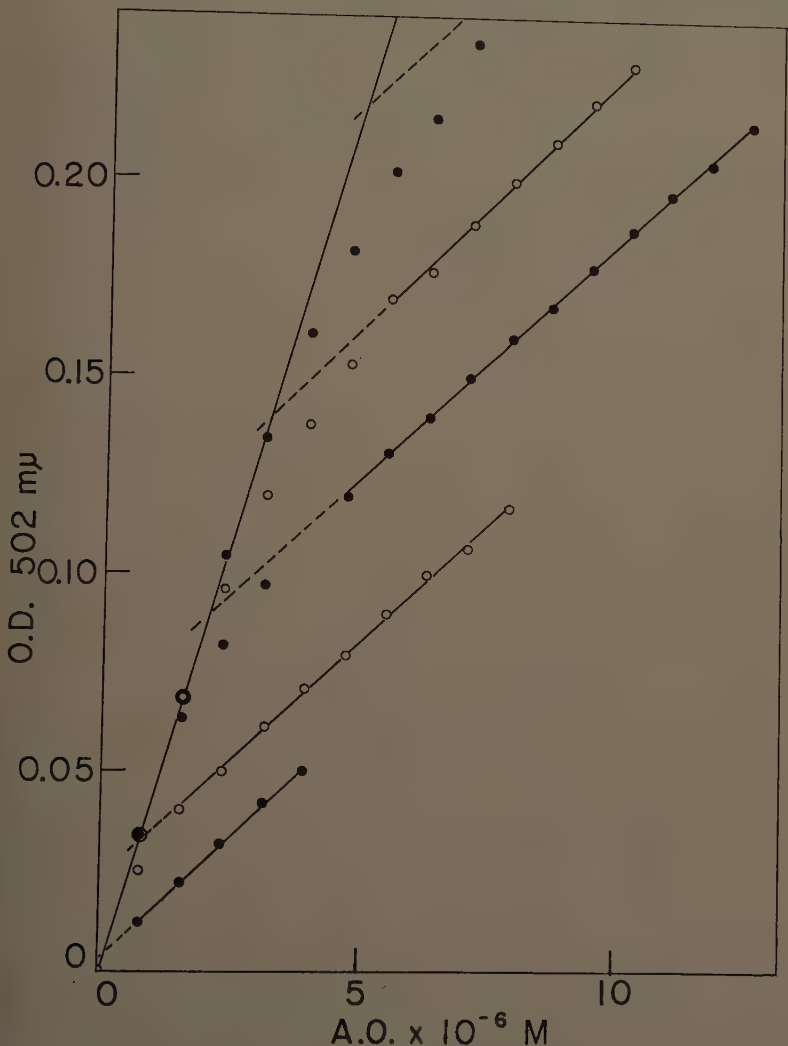

FIGURE 1. Titration of varying quantities of our poly A (No. 98) with acridine orange-$ZnCl_2$ in 0.01 M tris(hydroxymethyl)aminomethane, *p*H 8.0, at 20° C.

concentration of polymer and the amount of complex II formed with the Zn salt of acridine orange. In FIGURE 3 is plotted, as a function of KCl at *p*H 8.0, the concentration of acridine orange-$ZnCl_2$ required to saturate the complex II sites of a given polymer concentration. The quantity of dye bound is maximum at low and high KCl and has a minimum at about 0.1 M KCl; simi-

lar variations can be demonstrated with Na_2SO_4. This ionic variation is absent with Zn-free dye preparations, but it can be duplicated with the addition of Mg to the dye solution. Presumably, varying the ionic strength varies the ratio of Zn and dye binding by the complex II sites.

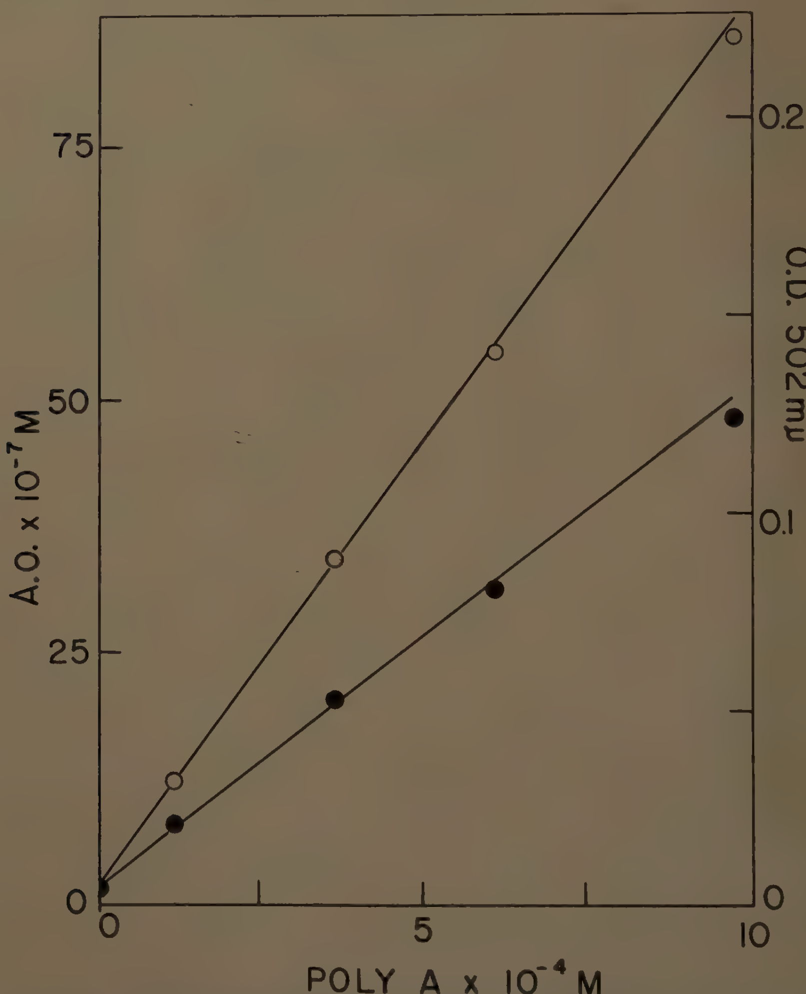

FIGURE 2. Symbols: ●, concentrations of acridine orange-$ZnCl_2$ bound to complex II sites as a function of poly A (No. 98) concentration; and ○, optical density increment from complex II formation with acridine orange-$ZnCl_2$ as a function of poly A concentration. Experimental conditions are the same as in FIGURE 1.

With increasing concentrations of Mg in the polymer solution, the amount of complex II formed decreases to nothing, irrespective of the amount of dye added. Direct displacement of the dye from complex II sites can also be demonstrated with the addition of Mg (Figure 1 in Beers *et al.*[6]). The competitive nature of the metal displacement of the dye can be shown by correla-

tion of the optical density increment observed with saturation of the "available" complex II sites by the dye with the concentration of dye required to saturate these sites. FIGURE 4 summarizes the results of a series of experiments in which the degree of saturation of the polymer by the dye was altered by varying KCl, $MgCl_2$, and Na_2SO_4. The slope drawn through the points is the extinction coefficient of complex II at 502 mμ. Thus, the apparent variation in "availability" of complex II sites appears to represent a competition of the dye with the metal for these sites.

A second effect of ionic strength is the appearance of a third species of dye complex. FIGURE 5 shows a series of titration curves, with the Zn salt of the dye at *p*H 8.0 in 0.2M KCl. A plot of the quantity of dye required to saturate the complex II sites as a function of polymer concentration reveals the same

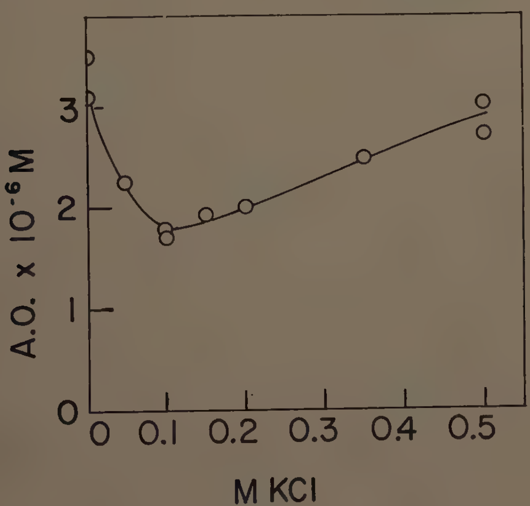

FIGURE. 3. Variation of the amount of complex II formed with poly A and acridine orange-$ZnCl_2$ as a function of KCl. Poly A concentrations: 6×10^{-4} M, 0.01 M tris(hydroxymethyl)-aminomethane, *p*H 8.0, 20° C.

linear relationship shown in FIGURE 2, but the intercept at zero polymer concentration is greater than zero. This blank value for the dye increases with ionic strength and Mg concentration (FIGURE 6). A plot of the optical density increment against the dye concentration at zero polymer concentration reveals an extinction coefficient equal to that of the "free" dye (FIGURE 7). The exact significance of this phenomenon is not clear, but it probably represents a complexing of the dye with either Zn or Mg; it is a substantial factor in the titrations of polyuridylic and polycytidylic acids. Complex I cannot be demonstrated with these polymers in the presence of high salt concentrations unless the concentration of dye is sufficient to overcome the apparent competition with the metal.

Poly A synthesis studies. Earlier studies have shown that polyase preparations from *M. lysodeikticus* require no primer and are very sensitive to ionic strength.[9] With more highly purified enzyme preparations containing no de-

tectable RNA (ϵ 280/260 mμ = 1.7)[12] no primer requirement could be demonstrated unless the ionic strength was sufficiently high to produce substantial inhibition. It is of particular interest to determine by end-group measurements whether the preparations contain sufficient primer to initiate synthesis of poly A or whether they can synthesize poly A *de novo*.

In FIGURE 8 are shown the growth in the relative population of polymer molecules during polymerization and the apparent degree of polymerization of

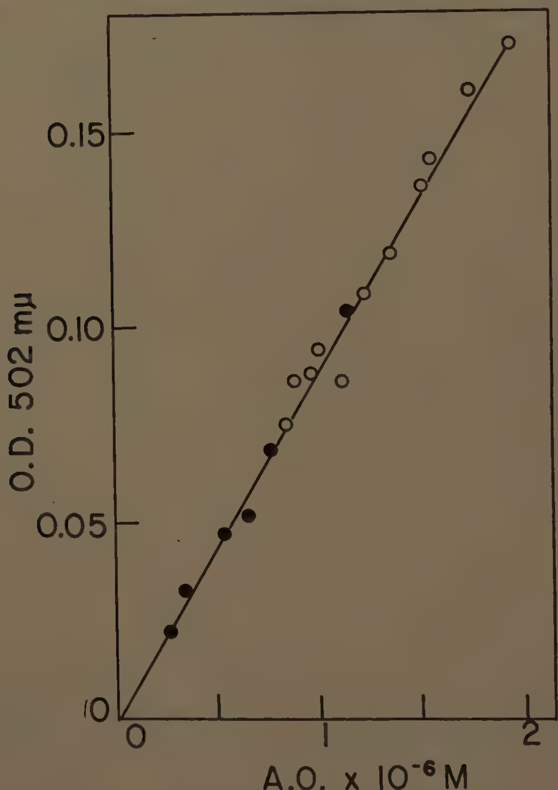

FIGURE 4. Optical density of complex II formed with acridine orange-$ZnCl_2$ and poly A (No. 98) as a function of the amount of dye required to saturate the complex II sites of poly A. Symbols: ○, KCl varied; and ●, $MgCl_2$ varied. Concentration: 0.01 M tris(hydroxymethyl)aminomethane, pH 8.0, 20° C.

the polymer, both at 2 concentrations of enzyme, 1 twice that of the other. During the first few seconds of the reaction, there is a sharp increase in the concentration of complex II from zero, followed by a slower rise that reaches a maximum at equilibrium. At equilibrium the concentration of complex II is the same for both enzyme concentrations, as is the degree of polymerization. Other experiments with fivefold variation in enzyme concentration have been performed in which the polymer was precipitated from the aliquot with 5 per cent $HClO_4$ titrated at pH 9.0; the results were identical. After 24 hours the concentration of complex II was the same, although the viscosity of the preparation had dropped to a very low value (Figure 7 in Beers[8]).

Since the rate of polymerization increases with ADP concentration it is possible that this results from either an increase in the population of molecules of polymer, which are substrate, in the rate of addition of monomeric units to the polymer, or in both. Plotted in FIGURE 9 are the equilibrium values for the

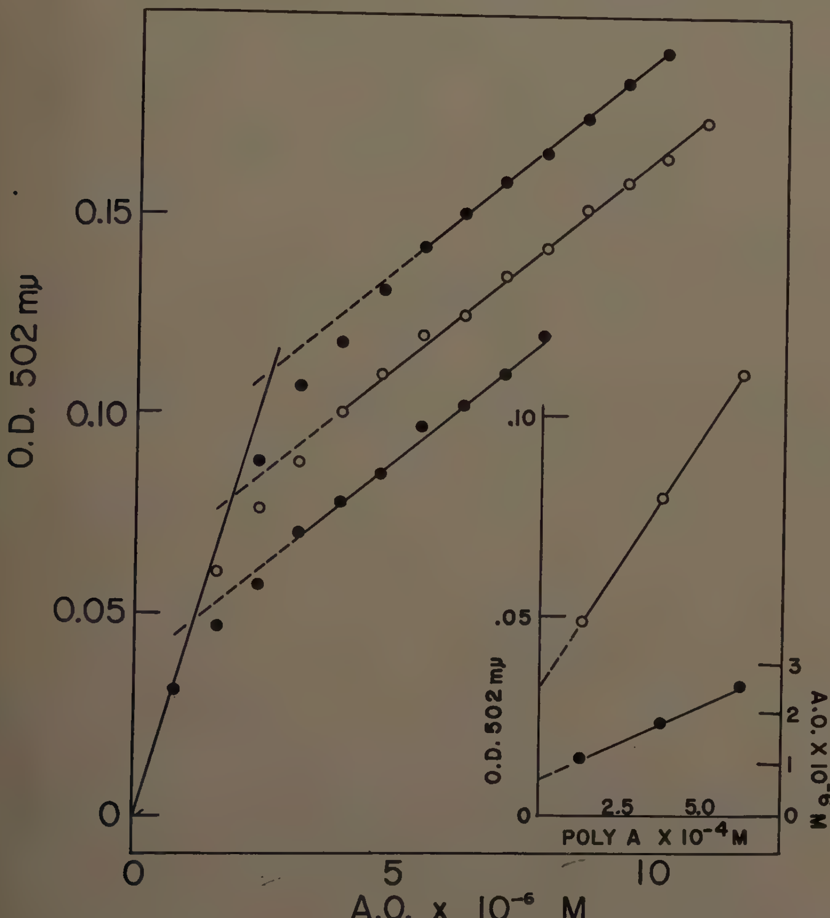

FIGURE 5. Titration of varying quantities of poly A with acridine orange-$ZnCl_2$ in 0.1 N KCl, 0.01 M tris(hydroxymethyl)aminomethane, 20° C. Symbols in inset: ●, concentrations of acridine orange-$ZnCl_2$ bound to complex II sites as a function of poly A (No. 98) concentration; and ○, optical density increment from complex II formation with acridine orange-$ZnCl_2$ as a function of poly A concentration.

mass of polymer synthesized, the concentration of complex II, and the apparent degree of polymerization as a function of ADP concentration. The amount of polymer synthesized is proportional to the initial concentration of ADP and accounts for 50 per cent of the nucleotide material. The concentration of complex II and the degree of polymerization increase with ADP concentration.

The activating effects of KCl and other salts on the polymerization and phosphorylysis of poly A[9, 13] are probably the result of the effects of the ionic

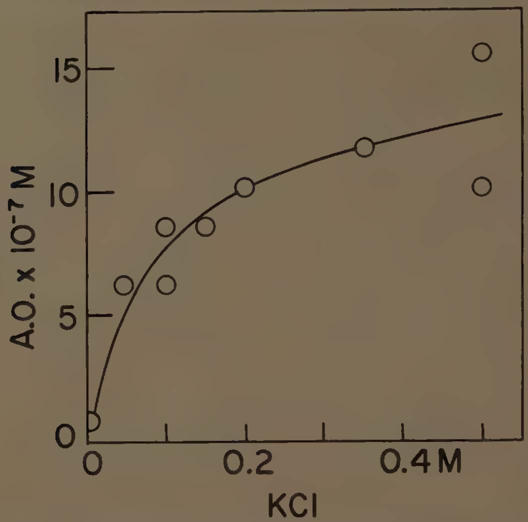

FIGURE 6. Variation of "free-dye" concentration with KCl in the titration of poly A (No. 98) with acridine orange-$ZnCl_2$ in 0.01 M tris(hydroxymethyl)aminomethane, *p*H 8.0, 20° C.

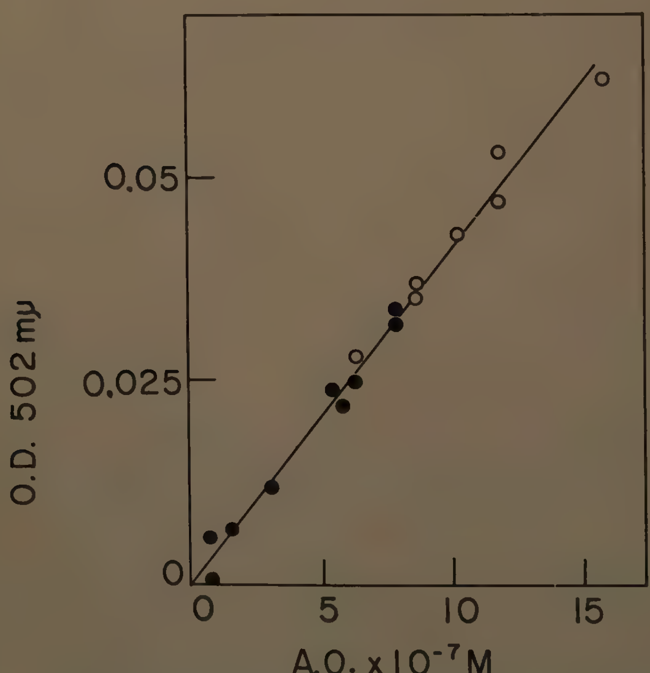

FIGURE 7. Optical density increment of "free dye" as a function of concentration of "free" dye. Symbols: O, varying KCl; ●, varying $MgCl_2$. Straight line is the extinction coefficient for acridine orange-$ZnCl_2$.

strength on the reactivity of the polymer. However, it is possible that the concentration of polymer molecules may also be sensitive to ionic strength. Two preparations of poly A were made with concentrations of ADP low enough to assure substantial activation by KCl (V_{max} is independent of the salt con-

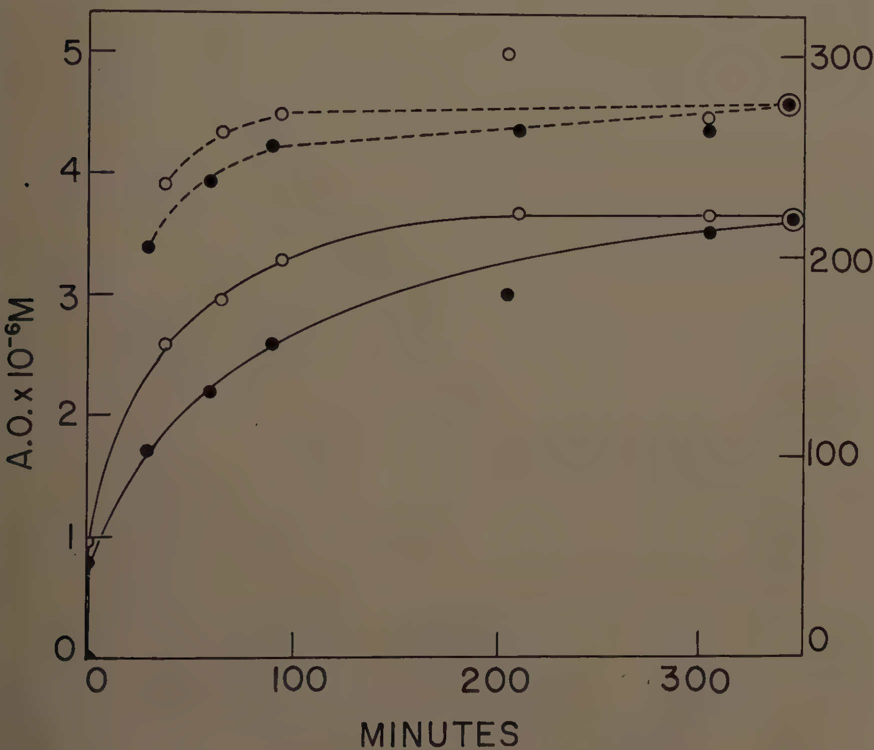

FIGURE 8. Time course of polymerization of poly A. Reaction mixture contained 3 ml. of 1 per cent ADP, 0.2 ml. 10^{-2} M $MgCl_2$, 0.5 ml. 0.5 M tris(hydroxymethyl)aminomethane (*p*H 9.5), 2.0 ml. H_2O, and 1 ml. of polyase preparation E-110. Aliquots of 0.5 ml. were removed at varying intervals and mixed with 2 ml. H_2O and 0.5 ml. 0.1 M acetate buffer, *p*H 7.0. The solution was titrated with acridine orange-$ZnCl_2$ at 20° C. Aliquots of 0.2 ml. were removed for inorganic phosphate determinations. Broken line represents the concentration of complex II, recorded in moles of acridine orange; the solid line, degree of polymerization. The enzyme concentration in the upper curve of the 2 pairs is twice that of the enzyme in the lower curve.

centration[9])—or 8×10^{-4} M. The concentration of complex II and the estimated degree of polymerization were identical in the 2 preparations.

The effects of Mg and *p*H also have been studied, but the results are equivocal because of the variations in yield of polymer material.

Discussion

The evidence that complex II formation is the result of a reaction between a terminal phosphate group and the dye can be summarized as follows:

(1) The stoichiometry of the complex II reaction compared with that of

complex I or total nucleotide composition of the polymer suggests a unique group characterized by its position at the end of the polymer chain.

(2) The increase in the concentration of complex II during the initial period of alkaline hydrolysis of poly A[6] and ribonuclease digestion of RNA[14] would

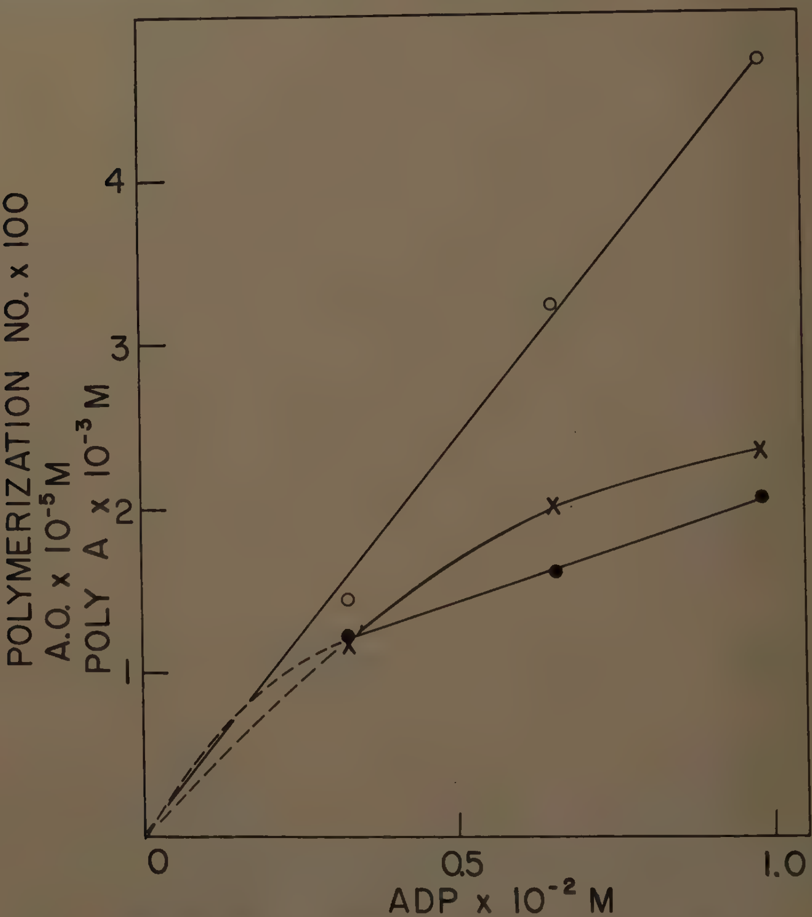

FIGURE 9. Mass, population, and degree of polymerization of poly A at equilibrium with varying initial concentrations of ADP. Experimental conditions are the same as in FIGURE 8, except that 0.5 ml. 2 M KCl replaced a portion of the water. Symbols: ○, concentration of nucleotide polymerized; ●, concentration of complex II; and ×, apparent degree of polymerization.

appear to reflect the increase in end groups resulting from random splitting of the polymers.

(3) The progressive decrease in the concentration of complex II during phosphorylysis of poly A by polyase is consistent with a terminal group splitting of the polymer by the enzyme.[15, 16]

(4) Brief treatment of poly A with phosphomonoesterase results in as much as 50 per cent decrease in the concentration of complex II.[17] This is followed

by extensive destruction of the polymer and a relative rise in the concentration of complex II. These findings are consistent with hydrolysis of the terminal 5′-phosphate group of poly A by the phosphomonoesterase and random splitting of the polymer chain by a diesterase contaminant.[18]

(5) The cationic dye is displaced from the complex II site by Mg and Zn, indicating that an anionic group is involved in the formation of complex II. The possibility that this anionic group is the terminal 2′-OH of the ribose is ruled out by the finding that DNA can also form this complex.[6]

The stoichiometric relationship of dye, metal, and complex II sites is complicated. It is beyond the scope of this paper to discuss this, although it is of interest to note that the displacement of the dye from the complex II site apparently requires 2 molar equivalents of Mg, 1 of which binds the complex II site, the other the dye. It is sufficient for our purposes to emphasize that under given conditions of ionic strength and *p*H, the fraction of complex II sites available to acridine orange (as determined by the titration procedure described above) is constant for each concentration of metal. The presence of Zn in the dye preparation does not alter this fundamental relationship. Therefore, for measurements of relative changes in population of molecules, either the Zn or Zn-free dye may be used. For maximum sensitivity, the *p*H of the titration should be above 9 and the salt concentration minimal, to reduce the extent of binding by the Zn.

The results presented in this paper indicate that polyase preparations from *M. lysodeikticus* can synthesize polymer molecules *de novo*. The failure to show any correlation between the amount of enzyme added and the final concentration of polymer molecules synthesized suggests that an absolute requirement for primer is not necessary; that is, each polymer molecule synthesized is not the result of adding monomeric units to preformed primer present in the enzyme preparation. The possibility that random splitting of polymer may serve as an autocatalytic primer-synthesizing mechanism is ruled out by the observation that the concentration of polymer molecules is constant for hours after equilibrium is reached. Moreover, the maximum rate of synthesis of new molecules appears to be at the beginning of the reaction, rather than later when the polymer concentration is maximal.

This enzyme preparation may contain a primer-synthesizing enzyme, but if this is the case it is difficult to understand why the primer synthesis reaction slows to a fraction of its original rate a few seconds after the reaction has started. Moreover, if an independent enzyme process were responsible for the synthesis of primer, it is difficult to understand why the synthesis of primer stops after equilibrium has been reached.

A single enzyme responsible for initiating the synthesis of primers and polymers could account for the observations described above. As pointed out elsewhere,[8] the polymerization and primer-synthesizing reactions can compete for the same enzyme. If the affinity of the enzyme is greater for the polymers than for ADP, with the synthesis of sufficient primer the distribution of enzyme-substrate complexes will favor the enzyme-primer complex over the enzyme-ADP complex. The major activity of the enzyme becomes a polymerization process. This argument is self-evident, of course, for any single enzyme system requiring no primer. If the enzyme favored ADP, dimerization rather

than polymerization would occur. Singer[19] has presented evidence that the affinity of the enzyme for the polymer increases with the degree of polymerization of the polymer.

Although one enzyme may be involved in primer and polymer synthesis reaction, the role of primers as an indispensable part of the enzyme preparation has not been ruled out. There remains the additional possibility that the primer acts as a template for the synthesis of additional primer. The relationship between primer synthesis and polymerization would be the same as above; however, the synthesis of primer would be autocatalytic. Some evidence for a template mechanism can be found in the results of Mii and Ochoa[20] and Singer *et al.*[21] These investigators have shown that highly purified preparations of polyase from *A. vinlandii* demonstrate a substantial lag in the synthesis of polynucleotides that can be overcome by the addition of polymer or oligonucleotides. Singer *et al.*[21] have shown that the primer added to the preparation is incorporated into the final product. This incorporation does not mean that each polymer molecule synthesized has arisen from a primer molecule. Significantly, these investigators have observed that the relative effectiveness of a given primer is dependent upon the composition of the primer and of the nucleoside diphosphates. For example, poly A will "prime" the synthesis of poly A and no other polymers. Synthetic "RNA" prepared by Ochoa from a mixture of nucleoside diphosphates and a polyase preparation from *A. vinlandii* had a base ratio similar to that of *A. vinlandii* RNA and different from that of the mixture of nucleoside diphosphates.[22] Recently, Heppel found that 3′-phosphate-ended oligonucleotides can act as primers, although they are not incorporated into the final product.[23]

The role of primers is complicated by the possible influence of inhibitors in the enzyme preparations. Grunberg-Manago *et al.*[24] have reported that RNA from *A. vinlandii* inhibits the phosphorylysis of poly A. Heppel *et al.*[23] have shown that 3′-phosphate-ended polymers inhibit phosphorylysis. In our laboratory we recently found that alkaline hydrolyzed poly A and commercial yeast RNA, both of which presumably contain significant amounts of 3′-phosphate-ended polynucleotides, inhibit strongly the polymerization of polynucleotides.[12] The extent of this inhibition depends upon the nucleoside diphosphate, requires a minimum concentration of Mg, and increases with increased salt concentration. Indeed, if a sufficient concentration of degraded polymer is present, the salt activation curve with KCl in the polymerization of polynucleotides[9, 13] is replaced by a progressive-inhibition curve. It may be significant that polyase preparations of *M. lysodeikticus*, which demonstrate marked salt activation, show no requirement for primer, even when the quantity of RNA in the enzyme preparation is too small to be detected; polyase preparations of *A. vinlandii*, however, which have not been reported to require salt for maximum activity, are primed by the addition of polymer, despite the fact that the purest preparations contain an estimated 3 per cent RNA.[20] Polyase from *M. lysodeikticus* can be activated as much as twofold by the addition of poly A, provided the concentration of salt is high and at a level that would be expected to increase the extent of inhibition by contaminating RNA. Indeed, fractionation of polyase with $(NH_4)_2SO_4$ from *M. lysodeikticus* extracts

will yield preparations with a total activity of 125 per cent or more of the activity present in the unfractionated material when the activity is determined as V_{max}, a constant independent of salt concentration,[9] which suggests that inhibitors are present that are removed by purification procedures.[25]

Because the population of polymer molecules is not influenced by subinhibitory concentrations of KCl, the effect of salt is presumably on the rate of polymerization. The decrease of the Michaelis constant and the constancy of V_{max} with increasing salt concentration[9] can be accounted for by a variation in the activity coefficient of the polymer. The fact that phosphorylysis of poly A also shows a similar response to salt[15] supports this thesis. However, because of the reciprocal relationship between salt and Mg,[9] the major effect may be on the relationship between polymer and Mg.

The number-average molecular weight of the poly A synthesized is primarily a function of the concentration of ADP, although we cannot be certain that this apparent "equilibrium" value of the number-average molecular weight represents a fundamental thermodynamic relationship of the end groups. The weight-average molecular weight, reflected in the viscosity changes,[8] continues to decrease long after the population and mass of polymers have reached constant values. Earlier studies[8] have shown that the magnitude of the viscosity increment is inversely proportional to the rate at which the polymer is synthesized. Fresco and Doty[26] have also shown that the weight-average molecular weight of poly A synthesized with polyase from *A. vinlandii* is inversely proportional to the enzyme-substrate ratio, although how this ratio was varied is not stated.

The decrease in weight-average molecular weight indicates a redistribution of the molecular chain lengths. At equilibrium, phosphorylysis and polymerization of the polymer continue at the same rate. Because the number-average molecular weight does not change, the phosphorylysis must occur only at the end of the polymer chain, a conclusion reached previously from kinetic studies[15] and demonstrated analytically by Singer[19] in the products of the phosphorylysis of oligonucleotides. However, the phosphorylysis of the larger polymers and the polymerization of the smaller polymers are faster than the phosphorylysis of smaller polymers and the polymerization of larger polymers. Therefore, the true equilibrium value for the weight-average molecular weight is not reached until the degree of polydispersity has been reduced to a smaller and constant value. This value should represent a true thermodynamic relationship.

Thus, both the number-average molecular weight and the weight-average molecular weight of the polymers are functions of the initial or, perhaps, under cellular conditions, steady-state conditions. Two kinds of "equilibria" are present in the *in vitro* system. The first reflects the *de novo* synthesis of polymer, and may represent an equilibrium between diphosphate nucleosides and inorganic phosphate and the dimer. Considerably more study must be made of the influence of the enzyme and reactants on this equilibrium before a distinction can be made between a true equilibrium and an apparent equilibrium resulting from a competition of the dimerization and polymerization reactions for the same enzyme. The second is a reflection of the relative reactivity of

polymers as a function of their chain length. Both the number-average molecular weight and the weight-average molecular weight would be expected to have considerable influence on the rate of exchange of phosphate with ADP.[24]

Summary

The reaction between acridine orange and poly A, leading to the formation of complex II, is believed to involve the terminal phosphate group of the polymer. The presence of Zn in the dye preparation results in a marked variation of the dye-binding capacity of the polymer as the ionic strength is varied. This, presumably, is caused by changes in the equilibrium distribution of the metal and dye on the complex II sites. A procedure for titrating the relative number of complex II sites of poly A has been developed with acridine orange. The estimations of relative number of complex II sites of various polymer preparations synthesized with polynucleotide phosphorylase from *M. lysodeikticus* indicate that the actual concentration of polymer molecules at equilibrium is independent of the enzyme concentration. Therefore, this enzyme system can synthesize polymer *de novo*, without the incorporation of a primer in each polymer molecule. Although varying the ionic strength of the reaction does not change the concentration of polymer molecules, varying the ADP concentration produces a corresponding change in the population and mass of polymer. The concentration of polymer molecules does not change after equilibrium is reached, indicating that phosphorylysis occurs only at the end of the chains. The progressive decrease in viscosity suggests that the larger polymers are being degraded whereas the smaller ones are being increased in length. Thus the number-average and weight-average molecular weights reflect the concentration of the substrate and time, respectively.

Acknowledgments

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THE EFFECT OF END GROUPS AND THE INITIAL SITE OF ATTACK ON POLYNUCLEOTIDES BY POLYNUCLEOTIDE PHOSPHORYLASE AND CERTAIN PHOSPHODIESTERASES

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Accompanying the increasing interest shown in the past ten years in the structure, the metabolism, and the functions of ribonucleic acids and desoxyribonucleic acids, a number of investigations have been made involving enzymes that hydrolyze these molecules. Although the functional role of these enzymes within the cell is a problem still to be solved, they have been very useful as analytical tools for elucidating the structure of polynucleotides. In addition to pancreatic ribonuclease and desoxyribonuclease, several phosphomonoesterases and phosphodiesterases have been used in the many efforts to define nucleic acid and polynucleotide structure. In recent investigations, the phosphodiesterases purified from snake venom[1-4] and from calf spleen[5] have proved very useful when employed in conjunction with other enzymatic and chemical methods.

In the first part of this presentation, these two phosphodiesterases will be discussed from the standpoint of their specificities, which contrast in an interesting way. It is noteworthy that the venom and spleen preparations hydrolyze both ribo- and desoxyribopolynucleotides; examples of their action on both types of compounds will be given.

Snake Venom Phosphodiesterase

As first shown by Cohn and Volkin,[6] this enzyme hydrolyzes ribonucleic acid (RNA) to form 5′-mononucleotides. As shown in FIGURE 1, the enzyme cleaves at the points indicated by the dotted line to liberate 5′-mononucleotides. It also hydrolyzes 5′-alkyl esters of mononucleotides, such as adenosine-5′-benzyl phosphate, but 3′-alkyl esters are resistant to attack. By the action of pancreatic desoxyribonuclease on desoxyribonucleic acid (DNA), desoxyoligonucleotides having 5′-phosphomonoester end groups are formed (FIGURE 2). Venom phosphodiesterase rapidly converts these desoxyoligonucleotides to the 5′-desoxymononucleotides.[4, 7, 8] Heppel *et al.*[9] found that biosynthetic polyribonucleotides containing a single type of nucleotide unit (poly A, poly U, and poly C) and those containing a mixture of nucleotide units[10] (poly AU and poly AGUC) are completely hydrolyzed to 5′-mononucleotides by the diesterase. Also, oligoribonucleotides, such as triadenylic acid (pApApA), shown in FIGURE 3, are rapidly split to 5′-mononucleotides,[11] as indicated by the dotted lines. Oligonucleotides having no phosphomonoester end group are also hydrolyzed by the venom fraction,[3, 4, 12] but at a slower rate. For example, triadenosine diphosphate (ApApA) is slowly split to adenosine and 5′-adenylic acid (5′-AMP), as shown in FIGURE 4. Khorana and his co-workers[13, 14] have reported similar results with chemically synthesized thymidine oligonucleotides having either the 5′-phosphomonoester group or no phosphomonoester group.

According to Volkin and Cohn,[12] oligoribonucleotides having 3′-phospho-

monoester end groups are resistant to the venom enzyme. Koerner and Sinsheimer[4] and Privat de Garilhe and Laskowski[3] have reported similar results with oligodesoxyribonucleotides having this end-group structure. The presence of a 2′,3′-cyclic phosphoryl group does not interfere with the activity.[15] The resistance of oligonucleotides having 3′-phosphomonoester end groups is not absolute, for if the concentration of venom fraction is increased by several orders of magnitude, hydrolysis does occur, as indicated in FIGURE 5. The

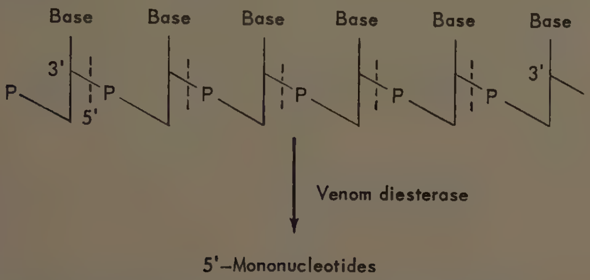

FIGURE 1. Hydrolysis of RNA by snake venom phosphodiesterase. The base may be purine or pyrimidine.

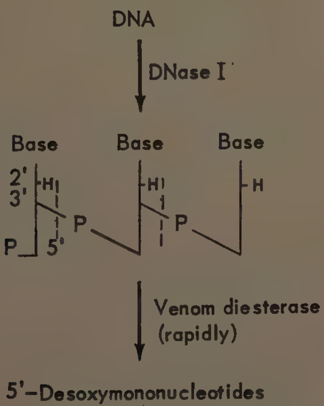

FIGURE 2. Hydrolysis by snake venom phosphodiesterase of desoxyoligonucleotides obtained from DNA by the action of pancreatic desoxyribonuclease. The base may be either purine or pyrimidine.

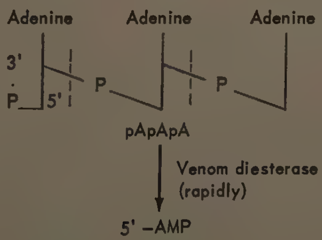

FIGURE 3. Hydrolysis of a 5-ended oligonucleotide by snake venom phosphodiesterase.

products formed are a nucleoside from one chain end, a nucleoside-3′,5′-diphosphate from the other end, and 5′-mononucleotides. Pyrimidine nucleoside-3′,5′-diphosphates were first isolated by Cohn and Volkin[6] from venom diesterase digests of RNA and of RNA that had been exhaustively digested with pancreatic ribonuclease (RNA "core"). Crestfield and Allen[16] were the first to propose that these diphosphates originated from nucleotide chain ends. With large amounts of venom diesterase, about 1 per cent of the nucleotides in a sample of RNA were liberated as diphosphonucleosides. When RNA "core" was digested with the venom enzyme, this value increased to 14 per cent. In addition to the pyrimidine diphosphonucleosides mentioned above, Crestfield and Allen[16] also isolated the purine nucleoside-3′,5′-diphosphates.

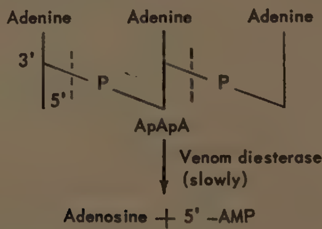

FIGURE 4. Hydrolysis of a trinucleoside diphosphate by snake venom phosphodiesterase.

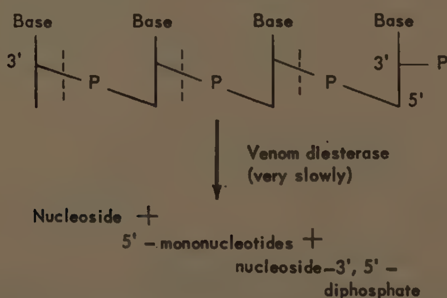

FIGURE 5. Hydrolysis of a 3-ended oligonucleotide by snake venom phosphodiesterase. The base may be either purine or pyrimidine.

Desoxyoligonucleotides having 3′-phosphomonoester end groups exhibit similar properties with this enzyme. Privat de Garilhe *et al.*[17] found that hydrolysis of desoxyadenylyl-desoxycytidylic acid (dApCp) to desoxyadenosine and desoxycytidine 3′,5′-diphosphate (FIGURE 6) required 1000 times more enzyme than was needed to split a similar "5-ended" compound, 5′-phospho-desoxyadenylyl-desoxycytidine (dpApC), to 5′-desoxyadenylic acid and 5′-desoxycytidylic acid. Further, among the desoxyoligonucleotides with 3′-phosphate end groups, resistance to venom was less for compounds with a longer chain.[4] To explain this observation, Koerner and Sinsheimer[4] suggested that a sufficiently long oligonucleotide chain could be broken at some intermediate link to form an oligonucleotide with no phosphate end group, such as the trinucleoside diphosphate of FIGURE 7 (*left*), and another with both 3′- and 5′-phosphomonoester end groups, such as the tetranucleotide pentaphosphate of FIGURE

7 (*right*). The oligonucleotide with no end-group phosphate would be readily degraded to a nucleoside and 5′-mononucleotides; the other, with both 3′- and 5′-monoesterified phosphate, would be hydrolyzed slowly to 5′-mononucleotides and, from the end of the chain bearing the 3′-monoesterified phosphate, a nucleoside 3′,5′-diphosphate.

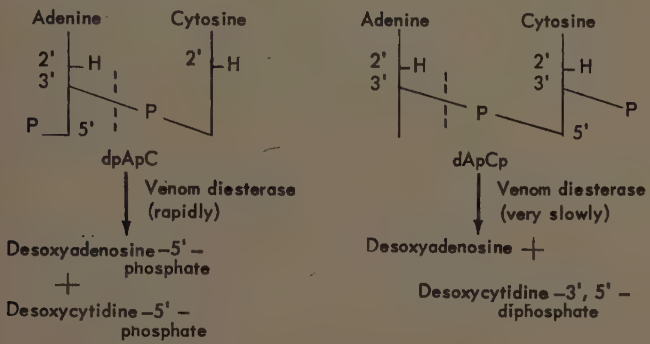

FIGURE 6. Hydrolysis of desoxyadenylyl-desoxycytidylic acid and 5′-phospho-desoxy-adenylyl-desoxycytidine by snake venom phosphodiesterase.

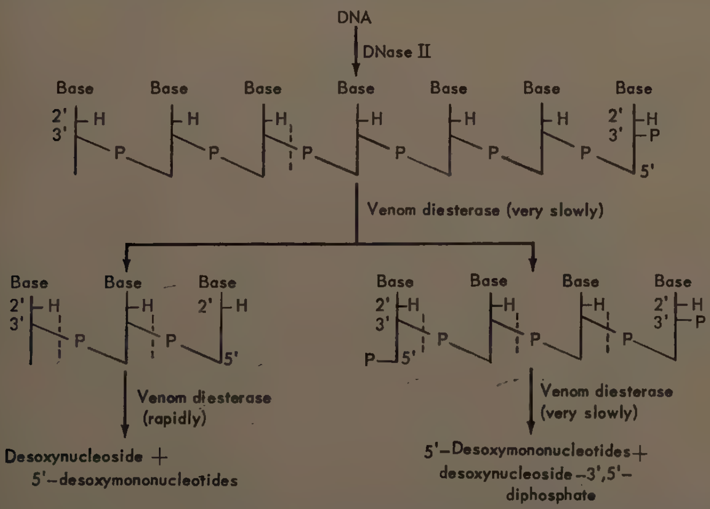

FIGURE 7. Hydrolysis by snake venom phosphodiesterase of a long oligonucleotide chain having a 3′-phosphomonoester end group. The base may be either purine or pyrimidine.

Because dinucleotides having a 5′-phosphate end group are hydrolyzed more rapidly than dinucleoside monophosphates, Laskowski[3] suggested that venom phosphodiesterase acts on the 5′-phosphomonoester end of a polynucleotide and removes 5′-mononucleotides by successive attack. Recent observations in Khorana's laboratory[13, 14] with a series of thymidine oligonucleotides have confirmed this hypothesis of a stepwise attack, but hydrolysis appears to begin, not at the 5′-phosphomonoester end of the chain, but at the opposite end. We

have confirmed these results with a labeled biosynthetic polymer and with the tetranucleotide pApApApG.

When the polymer prepared by incubating pApApA and uridine 5′-diphosphate (UDP) with polynucleotide phosphorylase—to extend the chain with uridine monophosphate (UMP) units (FIGURE 8)—was incubated with venom diesterase, only uridine 5′-phosphate (5′-UMP) was liberated in the initial stages of hydrolysis.[18] The action of the venom fraction on the tetranucleotide pApApApG also seems to indicate that hydrolysis occurs in a stepwise manner. The results of such an experiment are shown in TABLE 1. The products formed in the initial stages of hydrolysis were the trinucleotide pApApA,

pApApA + UDP

↓ Polynucleotide phosphorylase

pApApApUpUpU . . . . pU

↓ Venom diesterase

pApApApUpUpU . . . pU + 5′ –UMP

FIGURE 8. Formation of a labeled biosynthetic polynucleotide and the degradation of the polynucleotide by snake venom phosphodiesterase.

TABLE 1
STEPWISE HYDROLYSIS OF pApApApG BY SNAKE VENOM PHOSPHODIESTERASE*

Products formed	μMole
pApApA	0.023
pApA	0.002
5′-AMP	0.01
5′-GMP	0.02

* In a volume of 0.1 ml., 5 μmoles glycine buffer (pH 9.1), 1 μmole $MgCl_2$, 0.06 μmole pApApApG, and 0.036 mg. snake venom phosphodiesterase[4] were incubated at 37° C. for 6 hours. The mixture was applied to Whatman 3-mm. filter paper and the descending chromatogram was developed for 48 hours in the isopropanol-water-ammonia solvent system.[35] The products were quantitatively eluted and estimated by their ultraviolet absorption in a Beckman Model DU spectrophotometer.

the dinucleotide pApA, 5′-AMP and 5′-guanylic acid (5′-GMP). The small amount of pApA and 5′-AMP found, and the presence of nearly equal amounts of 5′-GMP and pApApA seem to indicate that the latter 2 compounds, pApApA and 5′-GMP, were the initial products of the reaction. The first step (EQUATION 1, FIGURE 9) in the hydrolysis of pApApApG would be cleavage at the bond indicated by the dotted line to form pApApA and 5′-GMP; pApApA would then be attacked at the indicated bond (EQUATION 2, FIGURE 9), with the formation of pApA and 5′-AMP.

Thus far, we have discussed the specificity of the enzyme mainly with regard to the nature of the phosphomonoester end groups present on various ribo- and desoxyribo-oligonucleotides. Another question is the size of the polynucleotides as related to their ability to act as substrates. The effect of molecular

size is very poorly defined. As mentioned earlier, with desoxyribo-oligonucleotides of longer chain length, the end-group specificity of the enzyme is not as pronounced as with compounds containing only a few nucleotide units.[4, 17] Possibly additional information concerning the requirements of this fraction could be obtained from studies of the rate of hydrolysis of several homologous series of nucleotide compounds having great variations in chain length.

When poly A and poly U are mixed, the polynucleotides interact through hydrogen-bonding between their adenine and uracil moieties[19] to form a double-stranded helix.[20] Ochoa[21] found that this poly A-poly U complex is phosphorylyzed by polynucleotide phosphorylase much more slowly than poly A or poly U alone. Similar studies with respect to the rate of hydrolysis of such complexes by the venom diesterase may also help define the substrate requirements of the enzyme.

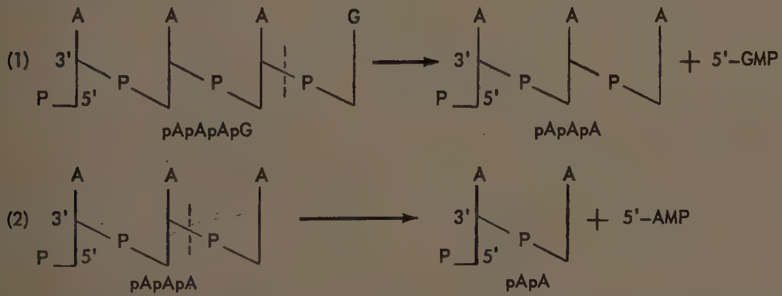

FIGURE 9. Stepwise hydrolysis of a tetranucleotide, pApApApG, by snake venom phosphodiesterase.

Spleen Phosphodiesterase

Spleen phosphodiesterase, which has been purified several hundredfold,[5] exhibits a specificity that is the opposite of that of the venom diesterase. It hydrolyzes RNA, RNA "core", and all nucleoside 3′-alkyl phosphates completely to 3′-mononucleotides. The 5′-alkyl esters are not attacked. Oligonucleotides formed by pancreatic ribonuclease digestion of poly AU have 3′-phosphomonoester end groups. These oligonucleotides are rapidly split to 3′-mononucleotides by the spleen fraction.[10] However, small "5-ended" oligoribonucleotides are not hydrolyzed.[11] Recently Koerner and Sinsheimer[4] reported that polydesoxyribonucleotides with 3′-phosphate end groups and an average chain length of 10 nucleotide residues are converted to 3′-mononucleotides by the spleen enzyme. The "5-ended" oligodesoxyribonucleotides formed by exhaustive digestion of DNA with pancreatic desoxyribonuclease are not attacked.

As mentioned earlier, the specificities of these 2 enzyme fractions from snake venom and spleen, with respect to end-group structure, are less sharply defined or are absent with large polynucleotide molecules. Hence, the biosynthetic nucleotide polymers that have 5′-phosphomonoester end groups are readily hydrolyzed by venom diesterase to 5′-mononucleotides and by spleen diesterase to 3′-mononucleotides.[9, 10] Neither of these enzymes exhibits any specificity with respect to the purine or pyrimidine bases.

Other Phosphodiesterases

Several other fractions that form 5′-mononucleotides from polynucleotides have been studied by Heppel,[22] who observed a striking effect of end groups with a fraction from hog liver nuclei. This fraction forms only 5′-AMP and 5-ended oligonucleotides from poly A, and only 3′-mononucleotides from RNA "core" and other oligonucleotides, such as adenylyl-uridylic acid (ApUp), bearing 3′-phosphomonoester end groups.

A guinea pig liver nuclei fraction splits poly A to form 5′-mononucleotides and 5-ended oligonucleotides.[11] A partially purified enzyme from an ascitic form of a mouse leukemia hydrolyzed poly A to 5′-AMP. RNA is also split, but at a slower rate. From HeLa cells, a fraction was separated that also acts on the synthetic polymers to release 5′-mononucleotides. No data are available on whether the mechanism of hydrolysis by these fractions is random or stepwise.

Thus, it is apparent that mammalian tissues provide several sources of phosphodiesterase activity that hydrolyze biosynthetic polymers and RNA to 5′-mononucleotides. Even in the rather crude preparations mentioned above, phosphomonoesterase activity is very low or absent. Cohn and Volkin,[23] in their early studies of RNA structure, used alkaline intestinal phosphatase to split RNA to 5′-mononucleotides. However, in this case arsenate was required to inhibit phosphomonoesterase activity and to prevent hydrolysis of the mononucleotides to nucleosides.

Nucleases

It is of interest to consider whether various nucleases active on RNA are affected by the nature of the phosphate end group. By and large, quantitative data regarding the action of these enzymes is not available. The pea leaf ribonuclease of Holden and Pirie[24] splits RNAs from several sources completely, producing nucleoside 2′,3′-cyclic phosphates.[25, 26] The purine nucleoside cyclic phosphates are slowly converted to 3′-mononucleotides, but the analogous pyrimidine compounds are not split by this enzyme. Similar results have been reported with poly A, poly U, and poly C.[25, 26] Reddi[27] has reported that tobacco leaf ribonuclease[28] hydrolyzes RNA just as the pea leaf enzyme does.

Sato and Egami[29] separated and partially purified 2 ribonucleases from takadiastase. One enzyme rapidly liberates 3′-guanylic acid (3′-GMP) from RNA, RNA "core", and poly AGUC, but the other 3′-mononucleotides were liberated very slowly, and poly A was attacked very little, if at all. The second fraction formed more 3′-adenylic acid (3′-AMP) and pyrimidine 3′-mononucleotides than 3′-GMP in the early stages of digestion of RNA.

Thus, we find there are nucleases that hydrolyze both RNAs and the 5-ended synthetic polyribonucleotides—poly A, poly U, poly C, and poly AGUC—to nucleoside 2′,3′-cyclic phosphates and 3′-mononucleotides. There are no data regarding the action of these enzymes (from pea leaf, tobacco leaf, and takadiastase) on 5-ended or 3-ended oligonucleotides such as pApApA and diadenylyl-uridylic acid (ApApUp).

The action of an enzyme of this type on small oligonucleotides with different end-group structures has been studied by Shuster *et al.*[25] Rye grass ribonu-

clease, like the pea and tobacco enzymes, hydrolyzes all phosphodiester bonds in RNAs, RNA "core," biosynthetic polymers, and oligonucleotides to nucleoside 2′,3′-cyclic phosphates. However, it differs from the pea and tobacco fractions in that both the purine and pyrimidine nucleoside 2′,3′-cyclic phosphates are finally hydrolyzed to 3′-mononucleotides.

The oligoribonucleotides used in the study with rye grass ribonuclease[25] included, among others, dinucleoside monophosphates and cyclic terminal dinucleotides. The dinucleoside monophosphates adenylyl-adenosine (ApA), adenylyl-uridine (ApU), and cytidylyl-cytidine (CpC) were each hydrolyzed to a 3′-mononucleotide and a nucleoside. The initial products of the reaction were the nucleoside 2′,3′-phosphate and the nucleoside. The nucleoside 2′,3′-phosphate was then hydrolyzed to the 3′-mononucleotide. The cyclic terminal dinucleotides uridylyl-uridine 2′,3′-phosphate (UpU > p), cytidylyl-cytidine 2′,3′-phosphate (CpC > p), and adenylyl-adenosine 2′,3′-phosphate (ApA > p) were each hydrolyzed with the formation of the cyclic intermediate, and the

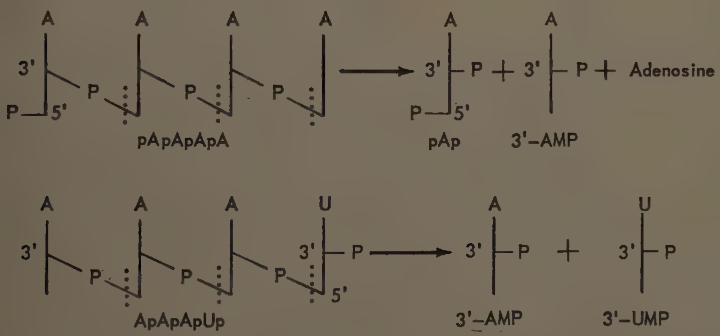

FIGURE 10. Hydrolysis by rye grass ribonuclease of oligonucleotides having 5′- and 3′-phosphomonoester end groups.

cyclic mononucleotides were further hydrolyzed to give the 3′-mononucleotides. The rate of splitting was slower for the cyclic terminal dinucleotides than for the dinucleoside monophosphates.

Two homologous series of oligonucleotides also were used in this study. These were the 5-ended di-, tri-, and tetranucleotides pApA, pApApA, and pApApApA, consisting of adenylic acid units, and the 3-ended di-, tri-, and tetranucleotides ApUp, ApApUp, and ApApApUp, consisting of adenylic acid units with a phosphate-terminal uridylic acid unit. All of the oligonucleotides were cleaved at every phosphodiester bond, with the formation of the terminal 2′,3′-cyclic phosphate, which was ultimately hydrolyzed to the 3′-phosphomonoester group. This is illustrated in FIGURE 10, using the tetranucleotide of each series as examples. Cleavage occurred at the bonds indicated by the dotted line. The final products from the 5-ended compound pApApApA were adenosine 3′,5′-diphosphate (pAp) from the terminal unit on the left, adenosine from the terminal unit on the right, and adenosine 3′-phosphate (3′-AMP) from the intermediate units. With the 5-ended dinucleotide pApA, only adenosine 3′,5′-diphosphate and adenosine were formed. From the 3-ended compound ApApApUp, only 3′-AMP and 3′-uridylic acid (3′-UMP) were

formed as the final end products. The rate of hydrolysis of these compounds was such that all possible intermediates, with both 2′,3′-cyclic and 3′-monoester terminal phosphate groups, were formed. Cleavage of the dinucleotides with 3′-phosphate or 2′,3′-cyclic phosphate end groups was slower than that of the corresponding higher homologues. However, the rates of hydrolysis of all the oligonucleotides studied did not vary more than twofold. Thus, in this case at least, the nature of the end group does not affect the structure of the products of hydrolysis, nor does it greatly alter the rate of hydrolysis.

No quantitative data are available regarding the effect of phosphomonoester end groups on pancreatic ribonuclease, but the enzyme certainly splits poly U and poly C to 3′-mononucleotides and small amounts of nucleoside and nucleoside 3′,5′-diphosphate.[9] Pyrimidine oligonucleotides with 3′-phosphomonoester end groups, such as triuridylic acid (UpUpUp), are completely hydrolyzed to 3′-mononucleotides, and those with no monoesterified phosphate, such as diuridylyl-uridine (UpUpU), are split to 3′-mononucleotides and a nucleoside.[22]

Polynucleotide Phosphorylase

Although polynucleotide phosphorylase is not a phosphodiesterase, it seems appropriate to point out how closely it resembles snake venom phosphodiesterase in its end-group requirements, initial site of attack, and mode of degradation of the polynucleotide chain when acting in the direction of phosphorolysis.

Polynucleotide phosphorylase, purified from *Azotobacter vinelandii* and *Escherichia coli*,[31] catalyzes the reversible reaction

$$n\ X\text{-Rib-P-P} \rightleftarrows (X\text{-Rib-P})_n + n\ P_i$$

In the forward reaction, nucleotide polymers are formed from nucleoside diphosphates, with the release of inorganic phosphate; in the reverse reaction, polyribonucleotides are phosphorolyzed to yield nucleoside diphosphates. Phosphorolysis of biosynthetic polymers and various RNAs has been studied by Ochoa and his co-workers[21, 30] and by Littauer and Kornberg.[31] They found that the synthetic polymers were more rapidly phosphorolyzed than the RNA preparations. In a study of the phosphorolysis of several homologous series of oligoribonucleotides, Singer[32] investigated the specificity of the phosphorolysis reaction. Oligonucleotides with 5′-phosphomonoester end groups are rapidly phosphorolyzed, but those having 3′-monoester end groups are resistant to enzymic attack. The 5′-phosphate group is not essential for activity, however, since oligonucleotides with no monoester phosphate, such as trinucleoside diphosphates, are phosphorolyzed. In addition, it was found that dinucleotides and dinucleoside monophosphates are not attacked by the enzyme, and accumulate as resistant end products when larger oligonucleotides are phosphorolyzed.

In addition to the question of the effect of end groups in this reaction, there is another one concerning the mechanism of phosphorolysis; that is, are long chains of polynucleotides attacked at an intermediate internucleotide bond or does the reaction proceed by stepwise release of mononucleotide residues from the end of the chain? Preliminary experiments have indicated that the latter is the case. In one experiment[33] the tetranucleotide pApApApA was incubated

with polynucleotide phosphorylase in the presence of inorganic phosphate, and aliquots of the reaction mixture were removed for chromatography in the isopropanol-water-ammonia solvent system.[35] If phosphorolysis occurred in the middle of the molecule as readily as at the end, one would expect approximately

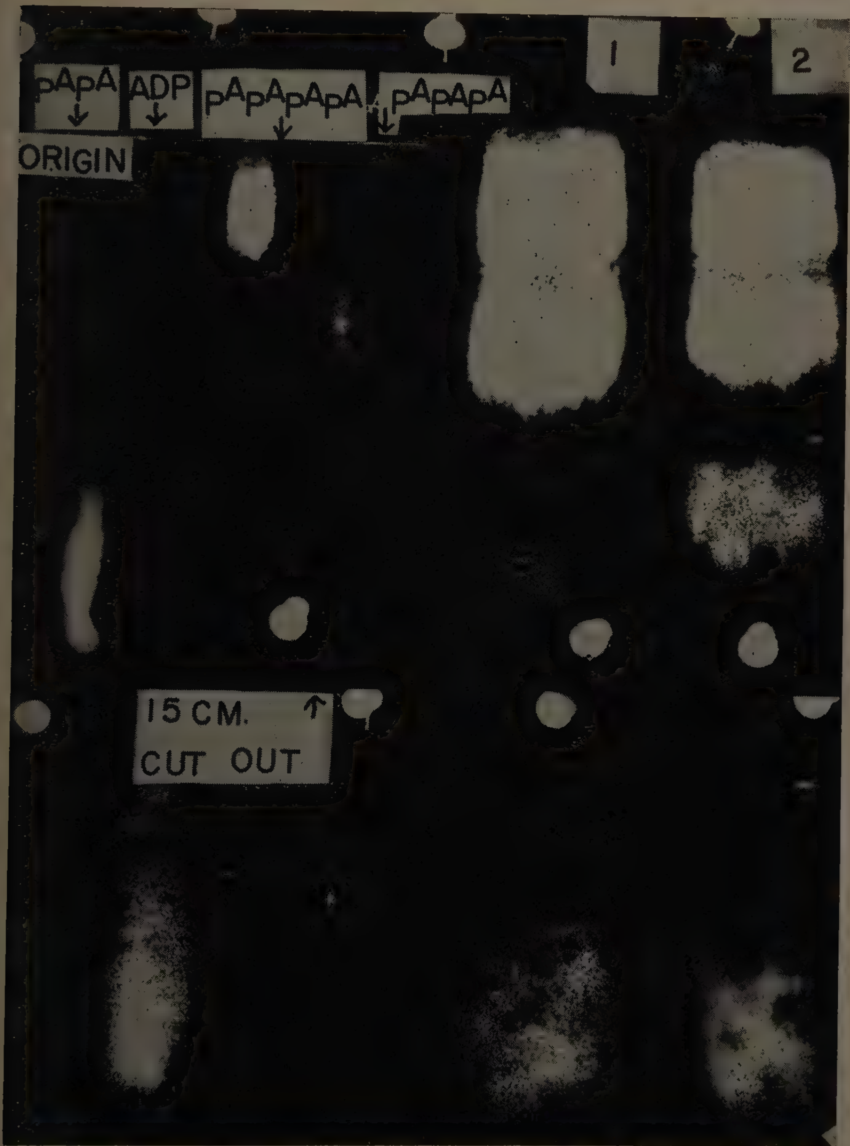

FIGURE 11. Phosphorolysis of pApApApA. In a volume of 0.2 ml., 10 μmoles Tris buffer, (pH 8.3), 1 μmole $MgCl_2$, 6 μmoles phosphate buffer (pH 7.2), 0.67 μmole pApApApA, and 0.004 mg. *E. coli* polynucleotide phosphorylase (specific activity 13.4) were incubated at 37° C. After 1 and 3 hours, 0.1-ml. aliquots were applied to Whatman 3-mm. filter paper and the descending chromatogram was developed for 48 hours in the isopropanol-ammonia-water solvent system.[35]

equal amounts of dinucleotide and trinucleotide to be produced initially. However, this is not the case (FIGURE 11). On the left of the chromatogram pictured in FIGURE 11 are markers of the dinucleotide pApA, ADP, the tetranucleotide pApApApA, and a very faint spot that is the trinucleotide pApApA. After 1 hour's incubation, Track 1, only a trace of dinucleotide, barely visible here compared to the relatively large amount of trinucleotide is formed. After 3 hours, Track 2, the dinucleotide area, shows an increase in intensity. The ADP produced migrates much faster than the oligonucleotides and appears near the bottom of the chromatogram. The appearance of large amounts of trinucleotide pApApA before significant quantities of dinucleotide pApA are formed suggests that phosphorolysis proceeds in a stepwise fashion (FIGURE 12) with the tetranucleotide being cleaved to form ADP and the trinucleotide pApApA, which is further phosphorolyzed to form a second mole of ADP and the resistant dinucleotide pApA. If one considers the relative rates of phosphorolysis[32] of the tetranucleotide and trinucleotide, it is not surprising that the trinucleotide accumulates.

In another experiment, with the tetranucleotide pApApApU as the substrate, similar results were observed.[34] Again, aliquots of the incubation mix-

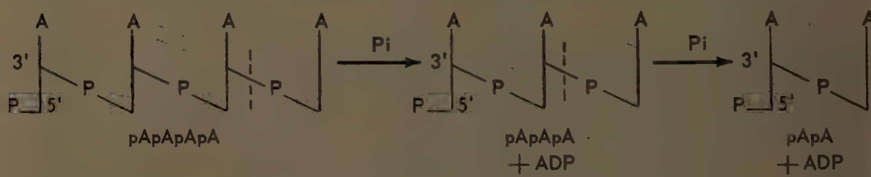

FIGURE 12. Stepwise phosphorolysis of a tetranucleotide, pApApApA, by polynucleotide phosphorylase.

ture were removed for chromatography. This chromatogram, developed in the isobutyric acid solvent system,[36] is shown in FIGURE 13 (*left*). Marker compounds shown on the extreme left of the chromatogram include UDP, ADP, and 5′-AMP. On the right is a marker of pApApApU that was retarded because of uneven solvent flow. After 1 hour's incubation, UDP and a trace of ADP were formed. In this solvent system the trinucleotide pApApA and the dinucleotide pApA have the same R_f. The dinucleotide-trinucleotide spot was eluted and rechromatographed in the *n*-propanol-ammonia solvent system;[37] this chromatogram is shown as the right half of the figure. In this case, the markers, on the right, are the trinucleotide pApApA, which is hardly visible in this photograph; the dinucleotide pApA; and ADP. After incubation for 1 hour only a trace of the dinucleotide pApA is found, but there is a much larger amount of the trinucleotide pApApA. Chromatography in the isobutyric acid solvent system after incubation for 3 hours shows that the amount of UDP formed is essentially unchanged, while the amount of ADP has increased. Also, after rechromatography of the trinucleotide-dinucleotide area, an increase is observed in the amount of the dinucleotide pApA and a slight decrease in the amount of trinucleotide pApApA. The tetranucleotide pApApApU, which has not been phosphorolyzed, is found just above the ADP after the initial chromatography. Again, the results suggest a stepwise phos-

phorolysis, as illustrated in FIGURE 14. The tetranucleotide pApApApU is phosphorolytically cleaved at the bond indicated to form UDP and the trinucleotide pApApA, which is further phosphorolyzed to form ADP and the resistant dinucleotide pApA. As a result of the apparent stepwise mechanism

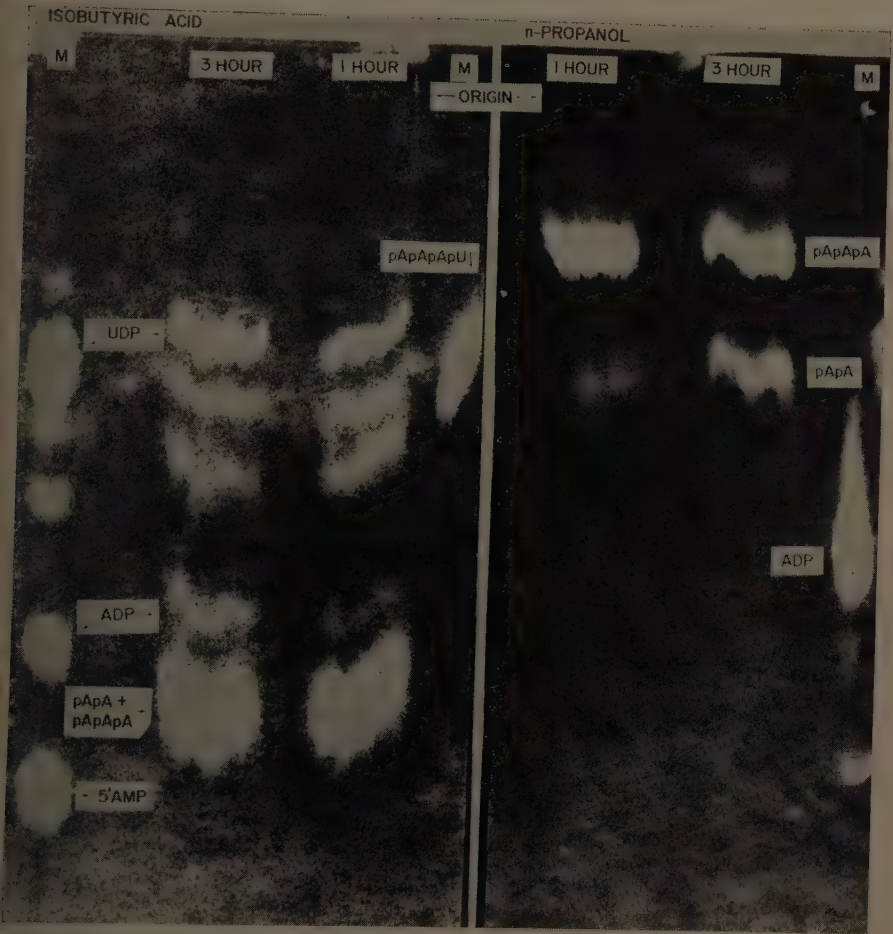

FIGURE 13. Phosphorolysis of pApApApU. Conditions were the same as described for FIGURE 11 except that the substrate was 0.335 μmole pApApApU. The initial chromatogram (*left*) was developed 24 hours in the isobutyric acid solvent system.[36] The dinucleotide, pApA, and the trinucleotide, pApApA, which have the same R_f in this system, were quantitatively eluted and rechromatographed on Whatman 3-mm. filter paper for 20 hours in the *n*-propanol-ammonia-water solvent system[37] (*right*).

of phosphorolysis and the products formed by complete phosphorolysis of oligonucleotides, it seems clear that the reaction represents reversal of the polymerization reaction. The terminal nucleoside residue attached to the polymer chain through a 5′-phosphate bond is phosphorolytically removed to form nucleoside diphosphate. Polynucleotides would be completely broken down by successive reactions of this type. It must be remembered that this

information on phosphorolysis was obtained using small, well-defined polynucleotides as substrates. Therefore, it does not necessarily follow that the results can be applied directly to phosphorolysis of high molecular-weight polynucleotides such as RNA and biosynthetic polyribonucleotides.

Summary

Snake venom phosphodiesterase rapidly hydrolyzes oligonucleotides with a 5′-phosphomonoester end group to give 5′-mononucleotides. Attack on compounds without an end group is slower. Oligonucleotides with a 3′-phosphomonoester end group are extremely resistant, but the resistance is not absolute. With oligonucleotides having either a 5′-phosphomonoester end group or no phosphomonoester end group, hydrolysis occurs in a stepwise fashion beginning at the end of the chain bearing a free 3′-hydroxyl. Both ribo- and desoxyribo-oligonucleotides are split.

Spleen phosphodiesterase shows an opposite specificity in that it rapidly hydrolyzes oligonucleotides with a 3′-phosphomonoester end group. It also acts more slowly on compounds with no end groups. Oligonucleotides having a 5′-phosphomonoester end group are resistant to attack. As with the venom

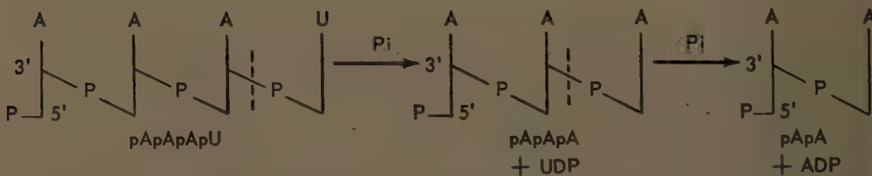

FIGURE 14. Stepwise phosphorolysis of a tetranucleotide, pApApApU, by polynucleotide phosphorylase.

fraction, spleen phosphodiesterase acts on both ribo- and desoxyribo-oligonucleotides.

Other mammalian tissues provide several sources of phosphodiesterase activity that hydrolyzes polynucleotides to 5′-mononucleotides. Specificity with respect to end-group structures is not known for these preparations.

The nucleases described hydrolyze all phosphodiester bonds of polynucleotides to form terminal 2′,3′-cyclic phosphates that are converted, for the most part, to 3′-monoesterified phosphates. The pea leaf and tobacco leaf ribonucleases do not split pyrimidine 2′,3′-cyclic phosphates to monoesterified phosphates. Rye grass ribonuclease acts on both 3-ended and 5-ended oligonucleotides to form nucleoside 2′,3′-cyclic phosphate intermediates, which are converted to 3′-mononucleotides.

Polynucleotide phosphorylase, in the phosphorolysis reaction, acts on oligonucleotides with a 5′-phosphomonoester end group and those with no monoesterified phosphate to liberate nucleoside diphosphate. Oligonucleotides with a 3′-phosphomonoester end group are not attacked. Phosphorolysis proceeds in a stepwise manner, beginning at the end of the chain having a free 3′-hydroxyl.

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MECHANISMS INVOLVED IN THE BIOSYNTHESIS OF RIBONUCLEIC ACIDS

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The cytoplasmic fraction of tissue homogenates obtained after high-speed centrifugation contains a special type of ribonucleic acid (RNA) of low molecular weight distinct from microsomal RNA.[1-3] In addition, this cell fraction also contains the enzyme or enzymes associated with the addition of specific nucleotides to the chain of this RNA.[4-6] Studies in this area have shown that the terminal nucleotide sequence being synthesized (FIGURE 1) appears to be adenylic acid–cytidylic acid–cytidylic acid, which is attached to the RNA chain.[4, 5]

This information was derived (FIGURE 2) as follows.[4] Doubly labeled adenosine triphosphate, that is, ATP labeled with C^{14} in the purine ring, as well as with P^{32} in the phosphate unit proximal to the ribose chain, was incubated with the enzyme system under study. At the end of the incubation, the RNA was isolated and hydrolyzed with alkali. Alkali acts on the sites indicated in FIGURE 2 yielding radioactive adenosine and P^{32}-labeled cytidylic acid. These results indicate that the labeled adenylic acid was terminal in the nucleotide sequence and that it was attached to a cytidylic acid residue. Analogous experiments performed with C^{14}-cytidylic acid, in the absence of ATP, have shown that the antepenultimate nucleotide is cytidylic acid.[5]

The reaction associated with the attachment of the terminal adenylic acid to the cytidylic acid of the RNA involves ATP as the direct precursor, with the consequent liberation of pyrophosphate (FIGURE 2). The basis for this statement is as follows: with ATP as precursor, pyrophosphate is formed during the reaction; furthermore, the reaction can be completely inhibited by the addition of exogenous pyrophosphate, and adenosine diphosphate (ADP) will not replace ATP.[5, 7, 8] Analogous evidence indicates that cytidine triphosphate is the precursor for the incorporation of cytidylic acid.[5, 8]

The RNAs whose synthesis has been thus completed appear to be used for a specific biological purpose. Current thought on the biological significance of these RNA molecules is that they act as carriers of amino acids in the processes of protein synthesis.[1, 9-14] In other words, an amino acid is attached to the 2′ or the 3′ position of the ribose of the terminal adenylic acid[15] to form an "amino acid-nucleic acid" compound (FIGURE 3); this has been postulated to act as a carrier of the amino acid from the cytoplasm to the microsomes. In the microsomes, the amino acid is then incorporated into the microsomal protein.

This very fascinating hypothesis cannot be unreservedly accepted as yet, because it is difficult to dissociate this reaction from a similar nonenzymatic reaction that also occurs.[16, 17] Proof of the scheme must wait until such "amino acid-nucleic acid" compounds can be shown to incorporate their amino acids in defined patterns into proteins of known amino acid sequence.

Although the various reactions described above have been found to occur

only in mammalian tissues, it may be assumed implicitly that similar reactions leading to the synthesis of functionally similar compounds occur in other types of cells. This is an assumption, of course, and remains to be proved. However, the transfer of the activated amino acid to an RNA of low molecular weight by enzyme systems of bacterial origin has been demonstrated.[11]

The characteristics of this aspect of RNA synthesis in mammalian tissues may be summarized briefly as follows: (1) the precursors are the nucleotide

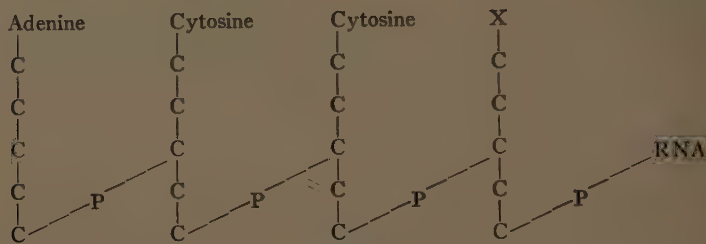

FIGURE 1

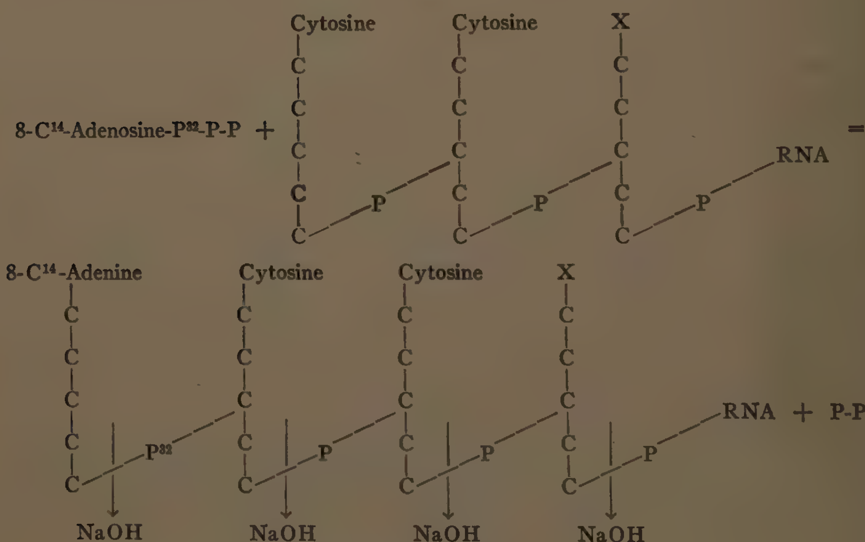

FIGURE 2

triphosphates; (2) the enzymes attach nucleotides in a specific sequence to an RNA chain; and (3) the products, that is, the amino acid-nucleotide compounds, appear to have a distinct biological activity.

The second of these characteristics, that is, the specificity of the enzymatic reaction, may bear further elaboration. This enzyme specificity that we have noted seems to indicate that the enzyme, alone or in association with the substrate, can define two things: (1) the nucleotide sequence; and (2), which may be more important, the site of the termination of the reaction, because the reaction stops and does not proceed further when the terminal adenylic acid is

added. In other words, the enzyme "knows when to stop." These experiments establish the fact that such an enzyme specificity, as well as an enzymatic delineation of the termination of the RNA chain, is possible, at least for a limited nucleotide sequence. This information may be well worth remembering as more polymerization reactions come to our attention.

Another feature of this general problem is the question of the net synthesis of RNA. From the properties of the enzyme system, as they have been described, it is obvious that this particular aspect of RNA synthesis is limited to reactions associated with the addition of specific nucleotides to an already existing polynucleotide chain. By definition, therefore, we can exclude the possibility of the attainment of net synthesis of RNA in this purified enzyme system, since the maximum synthesis that can be attained will be limited to the elongation, according to a defined pattern, of the RNA molecules that exist in or are added to the incubation mixture. Pursuing this trend of thought, further experimentation will lead to a more detailed understanding of an enzymatic reaction associated with the synthesis of specific nucleotides attached to an existent RNA chain but, most probably, it will not lead to the elucidation

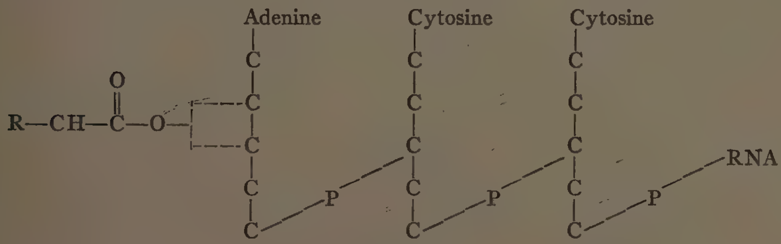

FIGURE 3

of the mechanisms of the *net* synthesis of RNA in animal tissues. Therefore, it is apparent that, in order to understand the problem of the net synthesis of RNA by mammalian tissues, a different approach must be taken.

In addition to these reactions, there exist in mammalian tissues other enzymes associated with the incorporation of nucleotides into RNA. These involve the incorporation into RNA[18, 19] of uridylic acid, as well as the nuclear polynucleotide phosphorylase,[20] which appears to have properties similar to those of the bacterial enzyme. Detailed investigation of these reactions may provide insight into the mechanism of the net synthesis of RNA by mammalian tissues.

Concerning the bacterial nucleotide phosphorylase, the isolation of the bacterial enzymes associated with RNA synthesis[21] has furthered our knowledge of the mechanisms that may be available for the net synthesis of RNA. This monograph contains excellent detailed expositions of the properties of this enzyme,[22, 23] and it is unnecessary for me to describe them at length. Suffice it to say, then, that a condensation of nucleoside diphosphates occurs to produce a net synthesis of nucleotide polymers, with the elimination of inorganic phosphate. This reaction provides an extremely useful mechanism for the biological synthesis of RNA.

Although certain nucleotide sequences are required to prime the reaction,

the characteristics of the resultant polymer depend on the substrates added to the incubation mixture, on the time of incubation, and on other externally imposed variables, but apparently not on an outstanding enzyme specificity.[22] Because of the lack of an ordered sequence of the type that we should like to find in biological polymers, a question must be raised concerning the biological significance of such polymeric compounds. It may be that in these bacterial systems an ordered sequence is imposed by the cellular organization of the polymerizing enzymes or by the existence of a defined template independent of these enzymes.

To summarize: we have today, on the one hand, mammalian enzymes associated with the addition of a specific polynucleotide to an existing molecule of RNA but apparently incapable of accomplishing any net synthesis of RNA. On the other hand, we have bacterial enzymes that can perform net RNA synthesis of RNA but that apparently lack the ordered specificity evidenced by the mammalian enzymes. An obvious question that may be raised is whether mixed RNA polymers of the *correct* chain length, synthesized by the bacterial polymerase, will act as substrates for the mammalian enzymes. Studies of such questions should help to disclose whether the correct sequence of nucleotide units is required along the entire polynucleotide chain or only at the functional end. Regardless of the outcome of such studies, the differences of these two systems will probably be explained or bridged by further experimentation.

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REACTIONS OF TERMINAL GROUPS OF RIBONUCLEIC ACID IN ANIMAL SYSTEMS*

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Reactions involving terminal groups of ribonucleic acid (RNA) have been observed in a number of cell-free systems from animal tissues (Zamecnik *et al.*,[1] Canellakis,[2] Paterson and Lepage,[3] Edmonds and Abrams,[4] and Herbert[5]). Studies with subcellular fractions prepared from liver homogenates[5] have shown that a system in the soluble enzyme fraction catalyzes the attachment of nucleotides to RNA as terminal groups, and that a system in the nucleus incorporates nucleotides into the interior of the RNA molecule. The incorporation of adenosine monophosphate (AMP) into RNA as a terminal group in the *p*H 5 enzyme system from ascites cells and liver has been shown to be dependent upon the presence of cytidine monophosphate (CMP) end groups.[6] This finding supports an earlier observation of Canellakis,[2] that AMP is incorporated into RNA as a terminal group adjacent to CMP. Interest in the mechanism of the terminal attachment of nucleotides to RNA has been heightened by the observation that the sequence RNA-CMP-AMP is essential for the transfer of radioactive amino acids to RNA in the *p*H 5 enzyme system.[7]

The *p*H 5 enzyme system[6] and the purified enzyme system to be described here attach a specific sequence and number of nucleotides to RNA. It is characteristic of these systems that, once AMP has added to the RNA, the reaction ceases whether or not other ribonucleotides are present in the medium. This is in contrast to the action of the bacterial enzyme, polynucleotide phosphorylase.[8, 9] The composition of the polymers formed by the latter enzyme in the absence of added primers appears to depend upon the relative concentrations of nucleoside diphosphate precursors present.[10, 11] Whether the latter system can be made to attach a specific number of nucleotides to a polynucleotide or primer chain (as in the present case) is not clear from the studies that have been completed.

Purification

Purification of the nucleotide-incorporating system was achieved by ammonium sulfate fractionation of the soluble enzyme fraction from rat liver, followed by adsorption on an hydroxylapatite column. Elution of protein from the column was accomplished by increasing the concentration of potassium phosphate buffer at *p*H 7. The details of the procedures used, including the preparation of radioactive CTP, UTP, and GTP, will be published elsewhere[13] and will be mentioned only when they are considered essential for interpreting the results. A summary of results of purification studies is shown in TABLE 1. It is apparent that ATP-C^{14} and CTP-C^{14} are incorporated into RNA much more rapidly than UTP-C^{14} and GTP-C^{14}.† It should also be noted that the

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† The concentrations of radioactive nucleoside triphosphates used here as precursors were those that gave maximum rates of incorporation of C^{14} into RNA when the concentrations

degree of purification attained is roughly the same at each step for CTP and ATP incorporation, suggesting that a single system is involved in the 2 cases. The second ammonium sulfate fractionation shown in the table was abandoned as a routine procedure, but proved useful in some instances because it reduced contamination by other enzymes.

The remainder of this report will be concerned with the results of studies carried out with the active fractions eluted from the hydroxylapatite column.

TABLE 1

ACTIVITY OF PARTIALLY PURIFIED FRACTIONS COMPARED WITH THAT OF THE SOLUBLE ENZYME FRACTION

Fraction	Radioactivity (moles × 10^{12} of precursor incorporated into RNA per milligram of protein)				$\frac{\text{mg. protein}}{\text{mg. RNA}}$	Recovery (percentage of activity)†
	ATP-C^{14}	CTP-C^{14}	UTP-C^{14}	GTP-C^{14}		
Soluble enzyme	7	10	—	—	133	—
Ammonium sulfate I (0.55–0.85)	31	39	4	2	90	85–90
Hydroxylapatite (0.2 M phosphate*)	550	720	25	8	29	80
Ammonium sulfate II (0.25–0.35)	3	2	14	—	30	30
(0.35–0.45)	500	660	26	—	18	18

* Fractions 3 and 4 of 0.2 M phosphate buffer.

† Of soluble enzyme fraction.

The soluble enzyme fraction in TABLE 1 was prepared by mincing rat liver with a scissors, homogenizing the mince in 2.5 times its weight of 0.35 M sucrose for 30 sec. in a Waring Blendor, and centrifuging the homogenate at 80,000 g for 45 min. The supernatant liquid was fractionated with ammonium sulfate to give the following degrees of saturation: 0 to 0.25, 0.25 to 0.35, 0.35 to 0.45, 0.45 to 0.55, 0.55 to 0.85. After dialysis overnight in 100 volumes of 0.02 M potassium phosphate buffer (pH 7.2), the active fraction (0.55 to 0.85) was placed on an hydroxylapatite column and the protein was eluted as shown in FIGURE 1. The fractions were assayed in a medium composed of 0.2 M potassium phosphate buffer (pH 7.2), 0.003 M $MgCl_2$, 0.004 M Na phosphopyruvate, and 0.05 mg. of pyruvate kinase. The protein concentration was 10 mg./ml. in the case of the soluble enzyme fraction; 5 mg./ml., for the first ammonium sulfate fractions (I); and 1 mg./ml., for the hydroxylapatite and second ammonium sulfate fractions (II). The concentrations of radioactive precursor used for the soluble enzyme fraction were 0.0002 M ATP-8-C^{14} (5 × 10^5 cpm/μmole) and 0.0001 M CTP-C^{14} (3.5 × 10^5 cpm/μmole); for the other fractions, they were 0.00004 M ATP-8-C^{14} and 0.00002 M CTP-C^{14}, UTP-C^{14} (4.3 × 10^5 cpm/μmole), and GTP-C^{14} (5 × 10^5 cpm/μmole). The volume of the incubation mixture was 3.0 ml. The assay was carried out for 6 min. at 25° C. The reaction was stopped by the addition of trichloroacetic acid, and RNA was prepared from the acid-insoluble fraction, as described.[5]

These results suggest that: (1) a single system is involved in the incorporation of CTP and ATP into RNA; (2) the integrity of protein and RNA is essential if the incorporation is to occur; (3) the nucleoside triphosphate is the immediate precursor of the terminal groups of RNA, and pyrophosphate is the product of the reaction; (4) under the conditions of study employed here, a specific sequence and number of nucleotides is attached to RNA; and (5) the nu-

were varied over a one hundredfold range. The GTP, UTP, and CTP were all uniformly labeled with C^{14}, while ATP was labeled with C^{14} in carbon atom No. 8. ATP-8-C^{14} was obtained from the Schwarz Laboratories, Mount Vernon, N. Y.

cleotide-incorporating system plays a role in the incorporation of radioactive amino acids into RNA.

FIGURE 1 shows in more detail the procedure used to elute protein from the hydroxylapatite column. The rates at which column fractions incorporate the 4 nucleoside triphosphates into RNA are given in the upper part of the figure; the lower portion gives the protein and RNA contents of each of the fractions; and the elution procedure is at the bottom. The correspondence

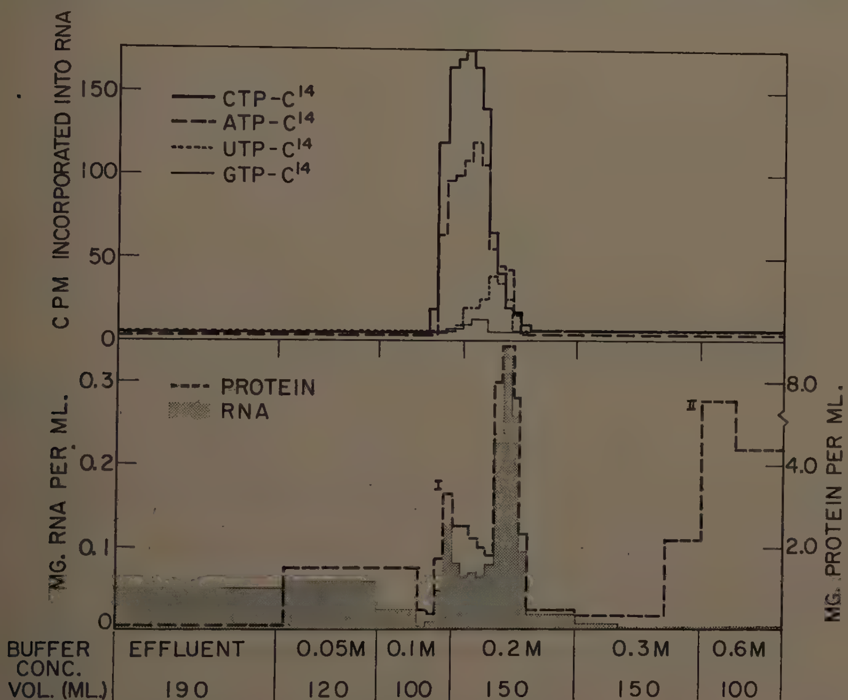

FIGURE 1. Elution of the nucleotide-incorporating enzyme from an hydroxylapatite column. Each step in the chart represents a fraction eluted from the column. The potassium phosphate buffer solutions were at *p*H 7.2. These solutions were added to the column as a 120-ml. portion for the 0.05 M buffer, as 10-ml. portions for the 0.1 and 0.2 M buffer, and as 25-ml. portions for the 0.3 M and 0.6 M buffer. The procedure used to measure incorporation of radioactive precursors into RNA was the same as that described in TABLE 1. The RNA content of the fractions was determined by an orcinol method,[5] and the protein content was determined by the method of Mokrasch and McGilvery.[14]

between the capacity of fractions to incorporate radioactive ATP and CTP into RNA is obvious. In view of the fact that only a small portion of the protein eluted from the column is in the active fractions, this result strongly suggests that a single system is involved in the incorporation. It should also be noted (lower part of figure) that the RNA has been fractionated into several components by this procedure. This is of some interest in another connection to be discussed later.

TABLE 2 shows that the 2 activities (ATP and CTP incorporation) are sensitive to the same chemical and physical treatment. In the absence of $MgCl_2$,

TABLE 2
EFFECT OF HEAT, $MgCl_2$, RIBONUCLEASE, AND TRYPSIN

Incubation system*	Precursor added	
	ATP-C^{14} cpm in RNA	CTP-C^{14} cpm in RNA
Experiment I		
Complete	84 76	116 108
-K phosphate	90 84	no result
-KCl	80 69	no result
-$MgCl_2$	18 12	10 10
Complete + 5 min. at 85° C.	6 1	8 1
Complete + 1 μg. ribonuclease	16 14	4 9

Experiment II

System*	Trypsin μg./ml.	Chymotrypsin μg./ml.	ATP-C^{14}	CTP-C^{14}
Complete	0	0	89 101	140 149
Complete	1	0	94 83	137 136
Complete	3	0	45 38	107 118
Complete	6	0	18 21	44 57
Complete	0	6	82 89	154 165

* Composition of the complete incubation medium in Experiment I was: 0.025 M KCl, 0.003 M $MgCl_2$, 0.004 M Na phosphoenolpyruvate, 0.1 M potassium phosphate buffer (pH 7.2), either 0.00004 M ATP-C^{14} (5×10^5 cpm/μmole) or 0.00002 M CTP-C^{14} (4.5×10^5 cpm/μmole), 0.05 mg. pyruvate kinase, and 1.0 mg. protein (hydroxylapatite fraction). The volume was 1.0 ml. Composition of 1.0 ml. of the incubation medium in Experiment II was: 0.1 M potassium phosphate buffer (pH 7.2), 0.003 M $MgCl_2$, and either 0.00004 M ATP-C^{14} or 0.00002 M CTP-C^{14}. In the cases where hydroxylapatite enzyme was treated with either crystalline pancreatic ribonuclease, trypsin, or chymotrypsin, the hydroxylapatite fraction was preincubated with the appropriate enzyme for 30 min. at 25° C. in 0.1 M phosphate buffer at pH 7.2; in the other cases, the hydroxylapatite fraction was preincubated with buffer alone for 30 min at 25° C.

there is almost no incorporation. Ribonuclease inactivates the system at a concentration of less than 1 μg./ml. The inactivation by mild heat treatment (5 min. at 85° C.) suggests that the integrity of a protein is necessary if incorporation is to occur. This interpretation is supported by the finding that a trypsin preparation, which has no detectable ribonuclease activity at a level of 18 μg./ml., almost abolishes incorporation in the 2 cases at a level of 6 μg./ml. Chymotrypsin has no effect on activity. Incorporation, then, is dependent upon the integrity of both protein and RNA. These results also show the typical variation encountered among radioactivity determinations on RNA from duplicate incubation mixtures.

The possibility was considered that the kind of pretreatment with ribonuclease shown in TABLE 2 may produce smaller acid-soluble fragments into which radioactivity is incorporated. Analysis of the acid-soluble fraction following pretreatment with ribonuclease gave no indication that this kind of incorporation occurs.

Formulation of the Reaction Pathway

The nucleoside triphosphate has been reported[2, 6, 13] to be a better precursor of the terminal nucleotide groups than either the nucleoside mono- or diphosphate. This observation and the finding that pyrophosphate inhibits incorporation of radioactivity into RNA[4, 6, 12] have led several workers[6, 12] to formulate the reaction pathway as follows:

$$\text{ATP} + \text{RNA} \rightarrow \text{RNA-AMP} + \text{P-P}$$

To determine whether this formulation is correct, a direct analysis of the reaction products was undertaken by incubating 10 mg. of protein from the second ammonium sulfate fractionation (TABLE 1) with ATP labeled as indicated in Reaction 1 (TABLE 3). One half of the incubation mixture was deproteinized with perchloric acid at 0 time, and the other half after 10 min. at 26° C. RNA was isolated and counted in the usual way, and the acid-soluble fractions were placed on Dowex-1 formate columns,[5] together with authentic samples of orthophosphate and pyrophosphate. The radioactivity eluted in fractions containing pyrophosphate and orthophosphate was determined,[13] and the result is shown in TABLE 3. Most of the P^{32} is liberated as pyrophosphate, establishing beyond reasonable doubt that the formulation shown above is correct. The orthophosphate release (TABLE 3) is believed to be caused by the action of pyrophosphatase, which is not completely inhibited by the level of fluoride used (Reaction 2, TABLE 3).

The Sequence and Number of Nucleotides Incorporated into RNA

AMP and CMP are incorporated into RNA in the *p*H 5 enzyme system in sequence, to give the terminal configuration RNA-CMP-AMP.[6] The following experiment was carried out to determine whether these nucleotides add to RNA in the same order under the conditions of study employed here. Two series of enzyme-incubation mixtures were prepared. In the first series CTP-C^{14} was incubated alone and in the presence of other nonradioactive nucleoside triphosphates. In the second series ATP-C^{14} was incubated in like manner.

RNA was isolated from the incubation mixtures, hydrolyzed with alkali, and placed on Dowex-1 formate columns together with authentic samples of nucleoside (either cytidine or adenosine) and nucleotide (either cytidylic acid or adenylic acid) as carriers. The elution was carried out as described in TABLE 4. The distribution of radioactivity in the nucleoside and nucleotide fractions eluted from the columns was determined, and the results are expressed in TABLE 4 as percentages of the total radioactivity in the RNA hydrolyzate. The results show that CTP-C^{14} is incorporated into RNA largely as a terminal group (67 per cent cytidine) when ATP, UTP, and GTP are omitted from the incubation mixture. The addition of ATP causes a pronounced shift in the proportion of C^{14} in cytidylic acid (from 24 to 84 per cent). The other nucleoside

TABLE 3

ATP AS PRECURSOR OF THE TERMINAL GROUP, AND PYROPHOSPHATE AS THE PRODUCT OF THE REACTION*

AMP incorporated into RNA (mμmoles)	P^{32}-P^{32} liberated (mμmoles)	P_i^{32} liberated (mμmoles)
350	235	90

* Reaction 1:

$$\text{AMP-8-C}^{14}\text{-P}^{32}\text{-P}^{32}\text{(ATP)} + \text{RNA} \rightarrow \text{RNA-AMP-8-C}^{14} + \text{P}^{32}\text{-P}^{32}$$

Reaction 2:

$$\text{P}^{32}\text{-P}^{32} \xrightarrow{\text{NaF}} 2\text{P}_i^{32}$$

The composition of the incubation medium used in TABLE 3 was: 0.003 M $MgCl_2$, 10 mg. protein from the second ammonium sulfate fractionation, 0.04 M NaF, and 0.2 μmole ATP, containing a total of 10,000 cpm of C^{14} and 600,000 cpm of P^{32} equally distributed between the second- and terminal-phosphate groups of ATP. The labeled ATP was prepared by incubating AMP-8-C^{14} with liver mitochondria and P^{32}-labeled orthophosphate under conditions of oxidative phosphorylation.[13] The incubation was carried out for 10 min. at 25° C. in a volume of 10 ml. The amount of radioactivity liberated as orthophosphate and pyrophosphate was determined by chromatographing aliquots of the acid-soluble fraction on Dowex-1 formate columns (15 × 0.78 cm.^{2}) before and after incubation, and by determining the radioactivity in the fractions in which authentic samples are eluted. The elution procedure is described in detail elsewhere.[13]

triphosphates, singly or in combination, have little effect. The simplest explanation of this result is that the enzyme adds nucleotides to RNA in an orderly manner (CMP followed by AMP). The results of incubation with ATP-C^{14} show that AMP-C^{14} is incorporated into RNA as a terminal group (100 per cent adenosine) whether or not other nucleoside triphosphates are added to the mixture.

The results in TABLE 4 can be summarized as follows: (1) CMP and AMP are incorporated into RNA in sequence to give RNA-CMP-AMP; (2) the system is specific for CMP and AMP; and, (3) once AMP has been incorporated into RNA, no further addition of nucleotides takes place.

The distribution of radioactivity in the alkaline hydrolyzate of RNA following incubation with CTP-C^{14} alone (67 per cent cytidine and 24 per cent cytidylic acid), makes it difficult to decide how many CMP molecules actually

add to each RNA molecule under the conditions of study employed. In view of the marked effect of ATP on the proportion of radioactivity incorporated into the cytidylic acid moiety of RNA (TABLE 4), it was thought that the presence of a small amount of ATP in the enzyme as it is isolated from the hydroxylapatite column might cover up sufficient CMP end groups, in the absence of added ATP, to give 24 per cent cytidylic acid. To test this possibility, enzyme was treated with activated charcoal before incubation to remove adsorbed nucleotides (ATP). Following removal of the charcoal by centrifugation and

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN THE ALKALINE HYDROLYZATE OF RNA

Additions		Radioactivity in hydrolyzate (percentage of total C^{14} in hydrolyzate)	
Radioactive precursor	Nonradoactive nucleoside triphosphate	Nucleoside	Nucleotide
CTP-C^{14}	none	67	24
CTP-C^{14}*	none	90	11
CTP-C^{14}	ATP	18	84
CTP-C^{14}	GTP	65	25
CTP-C^{14}	UTP	70	25
CTP-C^{14}	ATP, GTP, UTP	16	70
ATP-C^{14}	none	100	3
ATP-C^{14}	CTP	95	5
ATP-C^{14}	GTP	83	10
ATP-C^{14}	UTP	79	16
ATP-C^{14}	UTP, CTP, GTP	80	10

* Enzyme system pretreated with activated charcoal to remove nucleotides.

The composition of the incubation medium in TABLE 4 was: 0.003 M $MgCl_2$; 0.00002 M CTP-C^{14} (8×10^5 cpm/μmole) in the first series of incubations and 0.00004 M ATP-8-C^{14} (6.8×10^5 cpm/μmole) in the second; 1 mg. protein from the hydroxylapatite column; and 0.0004 M nonradioactive nucleoside triphosphate(s) where indicated. The volume was 1.0 ml. The incubation was carried out for 30 min. at 26° C. RNA was prepared from the incubation mixture in the usual way,[5] and, following hydrolysis in 0.1 *N* NaOH for 45 min. at 80° C., it was placed on a Dowex-1-formate anion-exchange column[6] (10×0.78 cm.^{2}) together with authentic samples of the nucleoside (either cytidine or adenosine) and nucleotide (either cytidylic acid or adenylic acid) as carriers. Six 10-ml. portions of water were used to elute the nucleoside, followed by eight 10-ml. portions of 0.05 *N* HCOOH to elute cytidylic acid. Adenylic acid was eluted with eight 10-ml. portions of 0.2 *N* HCOOH. Pretreatment with activated charcoal was carried out by adding 20 mg. of charcoal to the enzyme (10 mg. protein). Following 10 min. of stirring in an ice bath, the charcoal was removed by centrifugation and filtration through a celite pad.

filtration through a celite pad, the enzyme was incubated with CTP-C^{14}. The result shows that when adsorbed nucleotides are removed in this way, CMP is incorporated into RNA almost exclusively as a terminal group (90 per cent cytidine and 11 per cent cytidylic acid).

The addition of CMP to RNA as a terminal group, followed by the addition of AMP as postulated above, demands incorporation of 1 mole of CMP for every mole of AMP incorporated into RNA. Data taken from a series of experiments carried out over a 6-month period show that the average ratio of moles of CMP to moles of AMP incorporated into RNA is very close to 1 (1.05 in TABLE 5).

Role of the Purified System in the Incorporation of Amino Acids into RNA

The finding that the terminal RNA sequence CMP-AMP is essential for the incorporation of radioactive amino acids into RNA in the *p*H 5 enzyme system[7] raised the question of whether the partially purified system studied here plays a role in this phenomenon. In an attempt to determine the number of component systems involved in the incorporation of amino acids into RNA, the protein fractions eluted from an hydroxylapatite column (as previously described) were assayed for their ability to incorporate ATP-C^{14} into RNA and their ability to activate amino acids, since these activities apparently are necessary if incorporation is to occur in the *p*H 5 enzyme system.[7] FIGURE 2 shows that the 2 activities are completely separated by the procedure used here. The degree of amino acid activation is measured by the exchange of radioactivity between P^{32}-labeled pyrophosphate and ATP that results from the addition of a mixture of 10 amino acids (FIGURE 2). Exchange of pyrophosphate with ATP occurs with fractions from Peak II (FIGURE 2) in the presence of each of the following amino acids: leucine, valine, phenylalanine, tyrosine and, to a lesser extent, isoleucine, histidine, arginine, and tryptophan. It should be noted that Peak II in FIGURE 1, which corresponds to Peak II in FIGURE 2, contains no detectable RNA, whereas Peak I, which contains the nucleotide-incorporating system, contains RNA. Hence, RNA has been separated from the amino acid-activating system. Experiments were carried out in which RNA prepared by phenol extraction of the *p*H 5 enzyme system from rat liver (presumably free of enzymes) was compared with RNA in the nucleotide-incorporating system (Peak I) as an acceptor of radioactive leucine and

TABLE 5

MOLE RATIOS OF CTP TO ATP INCORPORATED INTO RNA*

Experiment	C^{14} Precursor ($\mu\mu$moles incorporated into RNA)		$\frac{\mu\mu \text{ moles CTP}}{\mu\mu \text{ moles ATP}}$
	CTP	ATP	
1	180	170	1.06
2	300	370	0.82
3	290	250	1.16
4	220	190	1.15
5	200	240	0.83
6	460	380	1.19
7	290	265	1.10
8	295	270	1.07
Average			1.05

* Figures were taken from experiments carried out over a 6-month period. Composition of the incubation medium was: 0.0003 M $MgCl_2$ 0.1 M potassium phosphate buffer (*p*H 7.2), either 0.00004 M ATP-C^{14} (6.8×10^5 cpm/μmole) or 0.00002 M CTP-C^{14} (8.0×10^5 cpm/μmole), 1.0 mg. protein from an hydroxylapatite column, 0.0004 M nonradioactive ATP when CTP-C^{14} was used, and 0.0004 M nonradioactive CTP when ATP-8-C^{14} was used. The incubations were carried out for 30 min. at 26° C. in a volume of 1.0 ml. The concentrations of the radioactive precursors used in all this work were those that gave maximum rates of incorporation into RNA in experiments on the relation of precursor concentration to activity.

valine. TABLE 6 shows that the nucleotide-incorporating system and amino acid–activating system are inactive when incubated separately; the combination of the 2 produces considerable incorporation. The RNA present in the nucleotide-incorporating system is apparently a better acceptor of amino acids

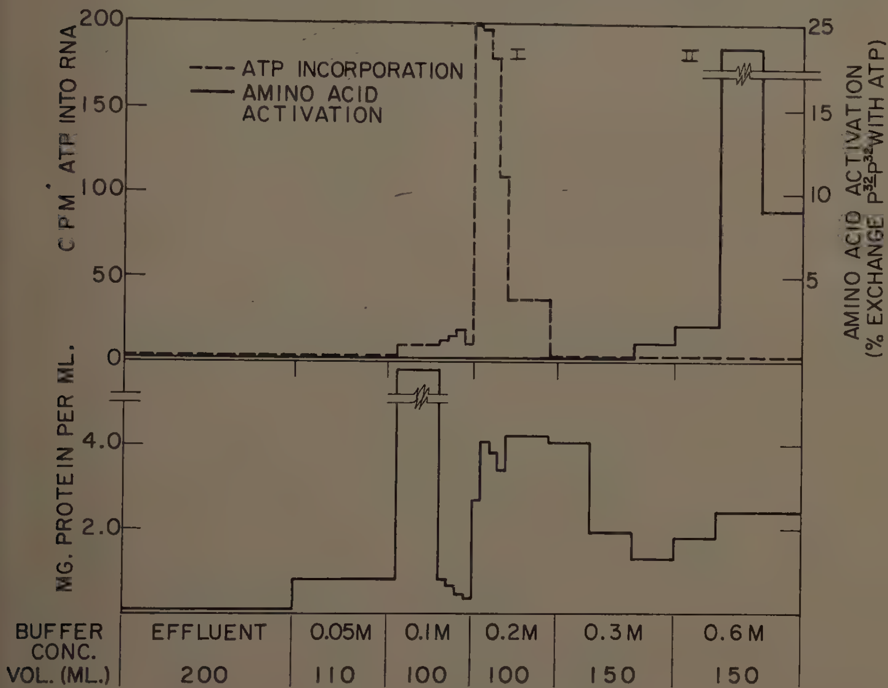

FIGURE 2. Separation of the nucleotide-incorporating system from an amino acid–activating system. The elution procedure used was the same as that described in FIGURE 1. The assay of ATP-C^{14} incorporation was carried out as described in TABLE 1. Amino acid activation was measured by incubating 1.0 mg. protein in a medium composed of 0.0025 M ATP, 0.1 M potassium phosphate buffer (*p*H 7.2), 0.05 M Na fluoride, and 0.0025 M P^{32}-P^{32} (0.6×10^5 cpm/μmole). Each fraction was incubated alone and in the presence of a mixture of 10 amino acids (0.002 M arginine, histidine, isoleucine, lysine, phenylalanine, threonine, tyrosine, leucine, valine, and tryptophan). After incubation, the mixture was deproteinized with trichloroacetic acid (TCA). Activated charcoal (150 mg.) was added to 5 ml. of the acid-soluble fraction (in 5 per cent TCA) to adsorb the nucleotides (ATP). The charcoal was washed 10 times with 25 volumes of 0.02 M pyrophosphate in 0.05 M K phosphate, and 2.0 ml. of 1.0 *N* HCl was added to it; the mixture was then boiled at 100° C. for 15 min. The charcoal was removed by centrifugation, 2.0 ml. of water was added to the supernatant liquid, and a portion of the sample was counted. The results shown in the chart represent radioactivity incorporated into ATP in the presence of the amino acid mixture, minus that incorporated into ATP in the absence of the amino acid mixture.

than *p*H 5 RNA. When the 3 systems are combined in the presence of CTP, phenol RNA apparently becomes as good an acceptor at low concentrations (to 100 μg./ml.) as the RNA in the nucleotide-incorporating system. This suggests that the latter system activates RNA from the *p*H 5 system. It should be noted, from Experiment II, that heating the amino acid–activating system destroys activity completely, while heating the other system to the ex-

tent known to destroy completely its nucleotide-incorporating activity (TABLE 2) reduces by only 50 per cent the incorporation of amino acid into RNA. These results suggest that activity of the nucleotide-incorporating system is not limiting for the actual transfer of amino acid to RNA. The picture that emerges from these results is one in which there are at least 3 components responsible for the incorporation of amino acids into RNA. Two of these are enzymatic; one activates amino acids, and the other attaches a specific sequence of nucleotides to RNA. The RNA so modified is the third component that actually takes part in the transfer reaction.

TABLE 6

INCORPORATION OF RADIOACTIVE LEUCINE AND VALINE INTO RNA OF THE pH 5 ENZYME SYSTEM AND THE NUCLEOTIDE-INCORPORATING SYSTEM

Amino acid-activating system	Nucleotide-incorporating system with RNA	Additions: pH 5 RNA μg./ml.	Additions: CTP μmoles/ml.	Radioactivity cpm/mg. RNA
		Experiment I		
+	0	0	0	30
0	+	0	0	100
+	+	0	0	4000
+	+	0	2	3900
+	0	100	0	1700
+	0	100	1	1900
+	0	100	2	1700
+	+	50	2	4350
+	+	100	2	3500
+	+	200	2	1050
		Experiment II		
+	+	0	0	5560
+*	+	0	0	0
+	+*	0	0	2600

* Preheated 10 min. at 85° C.

The composition of the incubation medium was: 0.003 M $MgCl_2$, 0.003 M Na_4ATP, 0.1 M potassium phosphate buffer (pH 7.2), and 0.00012 M leucine-C^{14} and valine-C^{14} (2.5×10^6 cpm/μmole). Protein (1.0 mg.) from Peak I and Peak II (FIGURE 1) was used. In the latter case there was 33 μg. of RNA present per milligram of protein. The incubation was carried out for 15 min. at 37° C. The volume was 1.0 ml. The RNA was isolated from the incubation mixtures in the usual way. The preparation of RNA from the pH 5 enzyme fraction by phenol extraction is described in detail elsewhere.[13]

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Part IV. The Structure and Properties of Synthetic and Other Polynucleotides

INTRODUCTORY REMARKS

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Polynucleotide Phosphorylase

Polynucleotide phosphorylase was discovered in 1955 in the course of studies by Marianne Grunberg-Manago and myself on the mechanism of formation of adenosine triphosphate in bacterial extracts. The existence of a new enzyme became apparent when we found that extracts of *Azotobacter vinelandii* catalyzed a rapid incorporation of radiophosphate into the terminal phosphate group of nucleoside diphosphates such as adenosine, guanosine, uridine, cytidine, and inosine diphosphate. Upon partial purification of the enzymatic activity, with use of the rate of incorporation of radiophosphate into ADP as an assay, the preparations were found to catalyze the liberation of orthophosphate from any of the above nucleoside diphosphates. The reaction produced an accumulation of a polynucleotide that could be isolated from the reaction mixture by precipitation with ethanol. The reaction catalyzed by polynucleotide phosphorylase can be written thus:

$$n\ X\text{-}R\text{-}P\text{-}P \rightleftharpoons (X\text{-}R\text{-}P)_n + n\ P$$

where R stands for ribose, P-P for pyrophosphate, P for orthophosphate, and X for one or more of the bases, adenine, guanine, uracil, cytosine, or inosine. The reaction requires the presence of Mg^{++} and is reversible. In the reverse direction it leads to the phosphorolysis of polyribonucleotides, with formation of the corresponding ribonucleoside diphosphates. For this reason the enzyme was named polynucleotide phosphorylase. When ribonucleoside diphosphates are incubated with polynucleotide phosphorylase, the liberation of orthophosphate comes to a standstill when 60 to 80 per cent of the easily hydrolyzable phosphate has been liberated; at this point the reaction has reached equilibrium.

Synthetic Polyribonucleotides

In the test tube, polynucleotide phosphorylase can give rise to a number of nonnaturally occurring polynucleotides. Thus, polynucleotides containing only one kind of nucleotide unit, such as polyadenylic acid (poly A), polyuridylic acid (poly U), polycytidylic acid (poly C), have been obtained. Copolymers containing two or more kinds of nucleotide units can be obtained by incubating the enzyme with mixtures of nucleoside diphosphates. A copolymer of adenylic and uridylic acid (poly AU) has been obtained in this way. Finally, when the enzyme is incubated with a mixture of the four naturally occurring ribonucleoside diphosphates, that is, adenosine, guanosine, uridine, and cytidine diphosphates, polymers are obtained that, like RNA, contain adenylic, guanylic, uridylic, and cytidylic acid.

Studies carried out in collaboration with L. A. Heppel on the chemical and enzymatic degradation of synthetic polyribonucleotides showed that these substances conform to the structural pattern of natural RNA and consist of chains in which the individual nucleoside units are linked to each other through 2′,3′-phosphodiester bonds. It has also been found that the polynucleotide (poly AGUC) synthesized from equimolar amounts of ADP, GDP, UDP, and CDP closely resembles the RNA isolated from *Azotobacter vinelandii* in base composition. Moreover, this polynucleotide is as effective as natural ribonucleic acid (RNA) in stimulating the formation of streptolysin by *Streptococcus hemolyticus*.

The molecular weight of polynucleotides synthesized by polynucleotide phosphorylase is of the same order of magnitude as that of natural RNA from various sources, varying from 30,000 to 2,000,000. X-ray diffraction studies by Alexander Rich and his collaborators have shown that polynucleotides containing at least one type of purine and one type of pyridine base in the molecule give diffraction patterns identical to those given by native RNA. It seems, therefore, that polynucleotide phosphorylase can bring about the synthesis of RNA indistinguishable from native RNA in structure, base composition, molecular weight, and X-ray diffraction pattern.

Polynucleotide phosphorylase is widely distributed in bacteria. There are also indications for the existence of this or a similar enzyme in yeast and animal tissues. It thus appears likely that polynucleotide phosphorylase is generally involved in the intracellular synthesis of RNA.

Reaction Mechanism

Purification of the *Azotobacter* polynucleotide phosphorylase in our laboratory has yielded preparations of approximately 70 per cent purity. In contrast to the synthesis of polynucleotides by crude preparations, that by the highly purified enzyme is very sluggish, with a pronounced lag period, and equilibrium is not reached even after prolonged incubation. Heppel will report on the finding that this lag is eliminated by addition of small amounts of certain oligonucleotides, such as triadenylic acid, which act as primers of polynucleotide synthesis. Heppel and his co-workers have found, in elegant studies, that the priming oligonucleotides serve as nuclei onto which nucleotide units are successively added. In this fashion, the oligonucleotide becomes an integral part of the newly formed polynucleotide chain to which it contributes the first few nucleotide units. The priming by oligonucleotides is not specific; thus, triadenylic acid will prime the synthesis of poly U as well as that of poly A. The capacity of partially purified preparations of the enzyme to synthesize polynucleotides, in the absence of added primers, may be due to contamination with primer molecules.

We found that the lag in polynucleotide synthesis with highly purified polynucleotide phosphorylase can also be eliminated by polynucleotides. The priming by polynucleotides is specific to a certain degree: thus, poly A will prime its own synthesis but not that of poly U or poly I, while poly U will prime its own synthesis but not that of poly A or poly I. None of the polynucleotides containing only one kind of nucleotide is able to prime the synthe-

sis of RNA. This, on the other hand, is readily primed by RNA, natural or synthetic.

The mechanism of priming by polynucleotides is not clear. It is unlikely that they function in the same manner as the oligonucleotides—that is, by having their chains lengthened through the addition of new nucleotide units. It is possible that they act like templates, causing a laying out of the growing polynucleotide chain alongside their own. Their priming specificity also suggests that polynucleotides may, indeed, act as templates for their own replication.

CONFIGURATIONAL STUDIES OF POLYNUCLEOTIDES AND RIBONUCLEIC ACID*

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About ten years ago the potential value of synthesizing and studying polypeptides of high molecular weight as simplified models of proteins was recognized. This led quickly to the improvement of synthetic procedures and to the preparation of a large number of polypeptides consisting of one or a few amino acid residues. The detailed examination of these polymers by X-ray, infrared, and other physicochemical methods made possible the selection of the α-helix from a number of other proposed structures as the only stable, folded configuration of the polypeptide chain. While this configuration was found to be dominant in a few proteins, such as tropomyosin, it was evident that in most globular proteins other forces operating between certain residues prevented more than a limited development of this helical structure. However, by using the pure helical forms found in the synthetic polypeptides to provide a precise calibration of certain sensitive physical methods, such as optical rotation and rotatory dispersion, the existence of helical regions embedded within the globular protein molecules was demonstrated and the fraction of residues composing such regions could be estimated.[1-3]

Now that about three years have elapsed since synthetic polynucleotides were made available by the discovery of their enzymatic synthesis by Grunberg-Manago and Ochoa,[4] it is appropriate to inquire to what extent the study of these polynucleotides can contribute to our understanding of the configurations of nucleic acids.

Of course, by the time the synthetic polynucleotides had been discovered, half of the problem of nucleic acid configuration had been solved by the general acceptance of the Watson-Crick structure for desoxyribonucleic acid (DNA).[5] Nevertheless, it was both exciting and instructive to learn from the observations of Warner[6] that polyadenylic acid (poly A) and polyuridylic acid (poly U), upon mixing in solution, formed complexes that were shown[7] to have the two-stranded helical structure of DNA. From this there have followed investigations of possible matings between various polynucleotide chains in solution.[8-11] A number have been found, although it is unlikely that all possibilities have yet been recorded. It is our aim here to examine the relative stability of some of these helical complexes and then to relate this to the unsolved half of the problem of nucleic acid configuration, the configuration of ribonucleic acid (RNA).

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Hypochromicity and Helix Stability

To deal in even a semiquantitative fashion with the helical structures that polyribonucleotides can assume in aqueous solution requires a ready means of detecting the structure in solution and a scale on which the stability of a given structure can be related to that of others. These two requirements have been met by the use of the hypochromicity of the helical complex relative to the separated polynucleotide chains and the melting or denaturation temperature of the helix.

It is well known that the extinction coefficient at the peak of the strong ultraviolet absorption band (near 260 mμ) is substantially less in DNA than in its constituent nucleotides. A corresponding increase in the extinction coefficient is observed when DNA is hydrolyzed. The hypochromicity of DNA is thus due to interactions that are possible when the nucleotides are held in close proximity to each other. It has been recognized more recently that a large part of the hypochromicity disappears when the helical structure of DNA is destroyed. Such destruction of the helical structure can be brought about by bringing the *p*H of the solution below 2.6 or above 11.9, or by raising the temperature of a low ionic strength solution above a certain temperature (70° C. for 0.001 M NaCl)[12] that depends on the ionic strength. Following any of these changes the extinction coefficient is found to increase about 40 per cent. A further but smaller increase is found upon hydrolysis to the mononucleotides, but this is not of interest in the present discussion. Our principal concern is that a 40 per cent increase accompanies the disappearance of the helical structure, since this suggests a way in which the helical structure in nucleic acid can be detected and, perhaps, semiquantitatively estimated.

A large hypochromicity is found in all the polynucleotide helical structures that have been discovered.[6, 8-11] Some specific examples of this are given below. Here we wish only to make the point that this hypochromicity is a general phenomenon, although the exact amount by which the extinction coefficient increases depends somewhat upon the particular nucleotides involved and the conditions of measurement. There has been considerable discussion as to the origin of this particular hypochromic effect; some argue that it arises from the interaction of the π-electrons of adjacent nucleotides, made possible through the stacking of the base groups in the helical structures, while others take the view that the effect resides in the distortion of the electron distribution caused by the existence of hydrogen-bonding. We tend to the latter view and, from surveying the known or most probable structures of the helical complexes thus far investigated, we have reached the tentative conclusion that the hypochromicity associated with chain configuration is due in large measure to the approach of a hydrogen atom to the N_1 position on the purine.

With this possibility of using the hypochromic effect for the estimation of helical content, we turn to explain the meaning of a melting temperature in assessing relative helix stability. The helical structures of DNA and the synthetic polynucleotides resemble one-dimensional crystals, since they do have periodicity in only one dimension. Consequently, when conditions are gradually changed in such a way as to make these helical structures unstable it is to be expected that their break down will have the character of the melting of

a crystal. That is, there will be a relatively sharp transition from the helix to an irregular chain structure known as a random coil. This transition should occur over a relatively narrow temperature range, the center of which can be denoted as the melting temperature, T_m. When comparisons are made in a given solvent for perfectly formed, high-molecular-weight helices, T_m should be a measure of relative helix stability.[12] Detailed study of such transitions has confirmed the expectation that they occur over relatively narrow temperature ranges when the melting is produced by raising the temperature. With the means at hand of following the transition in detail, it is possible to record in a routine manner the whole profile of the transition as a function of temperature. This provides not only an accurate assessment of T_m, but the breadth and asymmetry of the transition as well.

The discovery of these transitions and the recognition that they could be observed accurately has stimulated considerable theoretical investigation. This has now reached the point where it can provide useful interpretations of detailed features of the observed transitions.[13-18] For example, for polypeptides the variation of the transition profile with chain length has been nicely fitted and, therefore, adequately explained by the analysis of Zimm.[19] Previously, one had only the qualitative view derived from a general knowledge of cooperative phenomena. That is, with increasing chain length the transition would closely approach a step function, and T_m would have its maximum values. With decreasing chain length, however, the distribution would broaden and T_m would diminish. The theory provides a quantitative description of this dependence on chain length. Moreover, the assignment of values to the parameters involved shows that the probability of forming the first turn of the helix is substantially less than that of forming the following ones, thereby contributing to the sharpness of the transition.

In these calculations, it was assumed that the helix is made of identical subunits, as in poly-L-glutamic acid, for example. Unfortunately, this is not generally the case, and heterogeneity among the subunits—as occurs naturally in proteins and nucleic acids—will always have a broadening effect on the transition. Although there is no general solution of this problem, the qualitative conclusion itself is significant.

In conclusion, T_m is a measure of the relative stability of a series of helices having comparable length and similar compositional heterogeneity. The transition broadens with decreasing helix length and increasing compositional heterogeneity.

Helix-Coil Transitions in Polyribonucleotides[12]

In contrast to the complicated issues raised by examining the helix-coil transition of DNA, the multistranded helical complexes that can be formed with the synthetic homopolynucleotides available from the Manago-Ochoa polynucleotide phosphorylase system[4] offer ideal experimental materials. Of their several aspects that have been studied by Warner,[6] Rich *et al.*,[7, 8, 10, 11] Steiner and Beers,[20] and ourselves,[9] we are concerned with only 2: what base pairings are possible in neutral saline solution, and what are the relative stabilities of these complexes. We must, of course, allow for many more possibilities than

the 2 that occur in the Watson-Crick DNA structure—that is, guanine-cytosine (G-C) and adenine-thymine (A-T). For example, Donohue[21] has found that 20 possible pairs of 2 nucleotides chosen from the 4 available will form satisfactorily, in terms of hydrogen-bond distances. The search for all possibilities is not yet complete, but the 4 shown in FIGURE 1 are, at least, representative. First, in order of increasing stability, we have polyinosinic acid (poly I + I + I),* which is being studied as an analogue of polyguanylic acid, since the latter cannot be made with polynucleotide phosphorylase. Next is the three-stranded helix poly A + I + I, and then the two-stranded helical complexes, poly C + I and poly A + U; the solvent is 1 M NaCl. The transitions

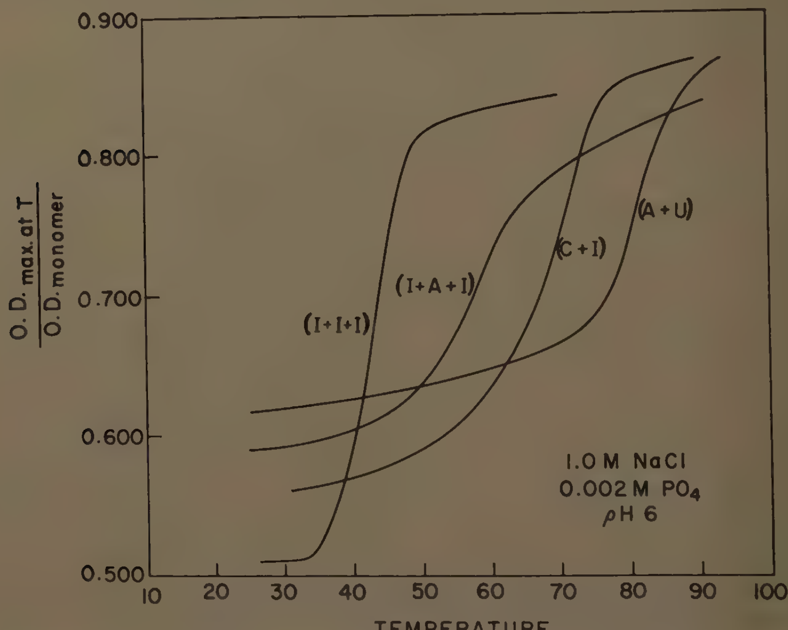

FIGURE 1. The variation in optical density as a function of temperature of solutions of helical polyribonucleotide complexes.

here are not as sharp as they might otherwise be, because structural defects have not been eliminated.

These examples demonstrate the fact that a variety of base pairings is possible and that helical structures incorporating these have stabilities in neutral saline solution ranging over most of the accessible temperature range.

Of the examples shown, the adenine-uracil (A-U) pairing is the most stable. In 0.15 M NaCl + 0.015 M sodium citrate, poly A + U has a T_m of 61° C. (FIGURE 6 below). The A-U pairing should be equivalent to the A-T pair that occurs in DNA. Since T_m for DNA in the same solvent lies at about 92° C., one might tentatively conclude that the G-C pairing is considerably more

* Polynucleotide complexes are indicated by the prefix *poly* followed by the summated homopolymer chain symbols.

stable, with a T_m in the vicinity of 120° C., so that the average of the 2 could account for the observed T_m of DNA. This possibility has been explored in another study and appears to be correct.[22] Consequently, the T_m for poly I + C should not be taken as substitute for that of poly G + C. Undoubtedly, the extra hydrogen bond that can form in the latter pairing accounts for the higher T_m. Accepting this conclusion, the T_m values for various pairings do cover the entire temperature range from room temperature to the boiling point.

Variations of ionic strength and *p*H obviously alter the transitions just described. Our study of the ionic strength effect shows that T_m is closely proportional to the logarithm of the NaCl concentrations. Increasing the *p*H sufficiently causes an increase in the negative charge and, hence, weakens the helical structure; in addition, it breaks up some of the bonded pairs. As a result, all of the helical complexes denature at alkaline *p*H. Upon lowering the *p*H from neutrality, the electrostatic charge is reduced and, if the titration does not interfere with hydrogen bonding, new helical complexes may form. This is clearly the case in poly A and poly C, both of which undergo self-pairing to form multistranded complexes below *p*H of approximately 5.5.[9, 23]

The Macromolecular Properties of RNA

Before attempting to relate the foregoing information to the problem of possible secondary structure in RNA, it is useful to summarize knowledge of the physical nature of RNA molecules in solution. Our studies have been with RNA carefully isolated from tobacco mosaic virus (TMV),[24] and from calf liver microsomal particles.[25, 26] For the former, the molecular weight is found to approach closely the maximum possible value of 2,100,000. In 0.02 M phosphate buffer the sedimentation coefficient, $s^{\circ}_{20,w}$ = 28 S, and the intrinsic viscosity, $[\eta]$ = 0.74. The sedimentation boundary for the most part was sharp, except for a pronounced tailing on the slow side.

RNA from liver microsomal particles prepared by the phenol method showed 2 separate boundaries in sedimentation, having $s^{\circ}_{20,w}$ of 28 S and 18 S in 0.02 M phosphate buffer. These could be assigned approximate molecular weights of 1,300,000 and 600,000. The relative amounts of each in the sedimentation pattern were inversely proportional to these weights, and 1 molecule of each would account for the RNA content of a single microsomal particle. In the same solvent $[\eta]$ = 0.41. Upon heating the solution to 85° C. and returning it to room temperature, the sedimentation pattern reduced to a single boundary of 8.2 S. This, combined with the new intrinsic viscosity of 0.22, indicated a molecular weight of about 120,000. These new properties are essentially the same as those found for RNA prepared by detergent methods. It therefore appears that the phenol-isolated material consisted of specific aggregates of RNA molecules that, perhaps, retained the intermolecular organization of the microsomal particle. Exposure to 85° C. permits a thermal dissociation into the constituent RNA molecules. Similar treatment of TMV-RNA has almost no effect.

We can learn to what extent RNA resembles a simple, single-chain polymer by determining how its sedimentation constant depends on molecular weight. This was done by using the TMV-RNA and the thermally dissociated microsomal RNA, as well as a number of degraded products of the latter. The

logarithm of their $s^{\circ}_{20,w}$ values is plotted against the logarithm of molecular weight in FIGURE 2. The result is seen to be strictly linear with a slope of 0.50. This is the result expected for a series of homologous, randomly coiled polymers having a considerable number of intramolecular contacts. The existence of such intramolecular contacts can be inferred from the observation that the intrinsic viscosities of these RNA samples are very low in comparison to what they would be for typical polyelectrolytes of comparable chain length at this relatively low ionic strength.

From this we can conclude that unaggregated RNA behaves hydrodynamically as homologous single chains that are coiled in such an average manner as to permit a substantial number of intramolecular bonds. These bonds are

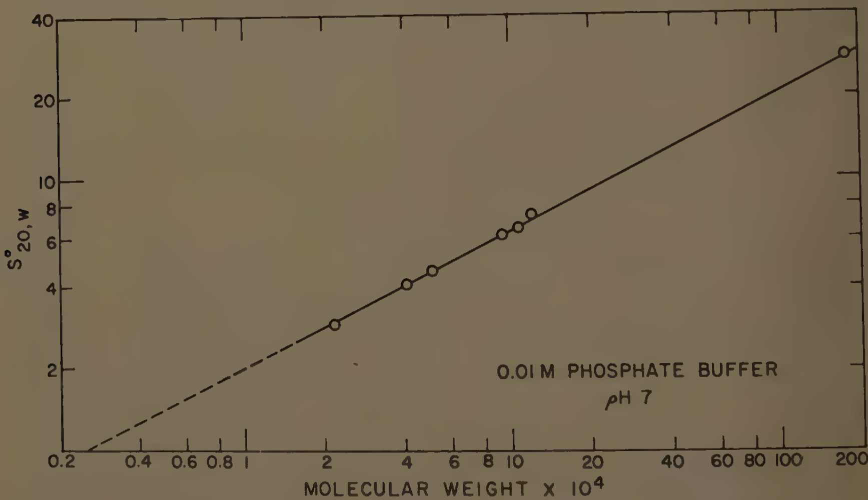

FIGURE 2. Sedimentation constant–molecular weight relation for ribonucleic acids.

sufficiently strong to prevent the expansion usually exhibited by polyelectrolytes.

The Variation in Hypochromicity of RNA[12]

The optical density at 258 mμ for TMV-RNA and microsomal-particle RNA as a function of temperature is shown in FIGURE 3. The total change relative to the minimum value is 32 per cent for TMV-RNA and about 22 per cent for the microsomal RNA. This compares with about 50 per cent for the average of the poly A + U and poly I + C complexes. The transition is somewhat sharper for TMV-RNA, 80 per cent of the change being accomplished over a 36° C. range in comparison with a 44° C. range for the liver RNA. These hyperchromic changes are reversible to the extent of at least 95 per cent, and their shift with ionic strength is similar to that for DNA and the polyribonucleotide complexes. The addition of 6 M urea causes one half the increase in optical density to occur at room temperature; the maximum is reached at about 70° C. Thus, the effect of the urea is to shift the curve to a lower tem-

perature by about 25° C. Urea, likewise, causes a lowering of T_m of DNA by about 18° C.

These parallelisms offer some support to the interpretation that the optical-density changes in RNA have their origin in the thermal dissociation of hydrogen-bonded base pairs. The gradualness of the transition is compatible with an arrangement of base pairs either into short helical regions or at ran-

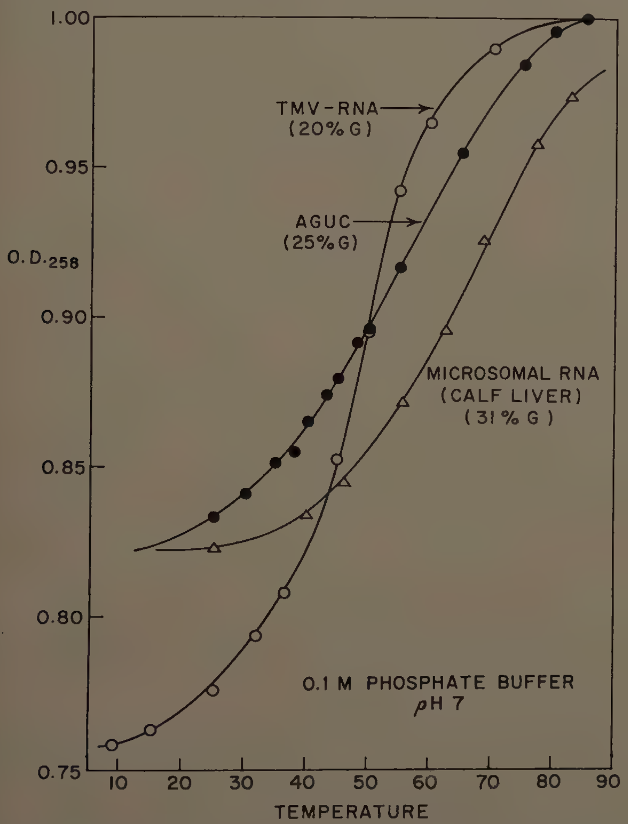

FIGURE 3. The variation in optical density as a function of temperature of solutions of synthetic and natural single-chain polyribonucleotides. The "% G" indicates percentages of guanine on a molar basis.

dom. However, the fraction of bases that must be paired (40 to 60 per cent, based on the optical-density changes observed in the complete helix-coil transition in the multistranded polynucleotide complexes) appears to be too high to be accommodated by any kind of random matching. Consequently, the hypothesis that RNA at room temperature does have base pairs arranged in helical regions is strongly favored. The broadness of the transition then could be explained by the factors cited earlier: relatively short helices, a distribution of helix lengths, a variation in average helix stabilities due to variations in the proportion of the stronger base pairs, and variations in the proportion of mis-

matched pairs incorporated in the helical regions. The optical-density temperature profile thus can be imagined as a composite of overlapping helix-coil transitions. It is important to note that the polynucleotide sample with the largest content of assumed helices and the sharpest transition, the RNA obtained from TMV, is the one in which the individual molecules are probably identical and in which, therefore, there is the least structural heterogeneity.

A question raised by this hypothesis is whether the assumed helical regions arise from specific sequences in the RNA molecules. As a test of this, an optical density–temperature profile was determined on a sample of a copolynucleotide composed of equimolar amounts of adenine, uracil, cytosine, and guanine. This profile, shown in FIGURE 3, is similar to those obtained for the natural RNA samples. Thus, specific sequences are not a prerequisite for this phenomenon.

A further point of interest concerns the T_m values of the apparent composite transitions. If the heterogeneity of TMV-RNA were increased to a point comparable with that of the other 2 samples in FIGURE 3, there is no doubt that the profile would broaden by a shifting of the lower part of the curve to lower temperatures. Such an adjustment, which would make the 3 curves approximately parallel, allows one to observe that their location on the temperature scale is proportional to the guanine contents indicated in FIGURE 3. This order is consistent with the assignment of the G-C bond as the strongest. It would be more nearly correct to assume that the number of G-C pairs would be proportional to the product of the mole fraction of G and C. This method predicts a similar ordering, the numbers being 0.047, 0.063, 0.102.

The Relation of RNA Reactivity to Hypochromicity[12]

Further evidence for the existence of hydrogen-bonded base pairs in substantial amount in RNA and for their dissociation with increasing temperature is found in the way in which the rate and extent of reaction of RNA depends on temperature. Three types of reactions reveal this relation: formylation, enzymatic phosphorolysis, and ribonuclease digestion.

Formaldehyde reacts with the amino groups of cytosine, adenine, and guanine; the reaction may be followed spectrophotometrically.[27] Hydrogen bonds involving these amino groups can be expected to retard the reaction and even limit its extent. This has been found to be the case for both liver RNA[26] and TMV-RNA. We note here only the observations made on TMV-RNA. The reactivity with formaldehyde at 25° C. and 45° C. is shown in FIGURE 4, where the difference in rate is seen to be about nineteenfold. A similar experiment with an appropriate mixture of mononucleotides showed only a sixfold increase in rate. Thus, the base pairing involving amino groups is manifest in the expected way.

RNA is phosphorolyzed in the presence of inorganic phosphate by the enzyme polynucleotide phosphorylase, producing nucleoside diphosphates. It has been observed that the rate of this reaction varies greatly with the type of RNA or synthetic polynucleotide employed.[28, 29] The completely hydrogen-bonded helical polynucleotide complexes were found to be the most slowly digested. In view of the argument presented here, a possible explanation of the phosphorolysis rates might be found in the fact that at any given tempera-

ture, including room temperature, different RNA samples exhibit a different degree of hydrogen bonding. Since this would impede the reaction, variation in rates at a common temperature would be expected. This proposal was tested by M. Manago in this laboratory by measuring the relative increase in rate of phosphorolysis at 40° C. over that at 25° C. for a number of different samples.[29] The relative increase in optical density at the 2 temperatures was also observed; these parameters were found to be linearly related, as shown in FIGURE 5, taken from her work.[29] Poly U does not exhibit base pairing; hence its optical density is independent of temperature. For poly U the rate of phosphorolysis is doubled only on going from 25° to 40° C., as compared to the much greater increases observed for the other samples. Thus we find here,

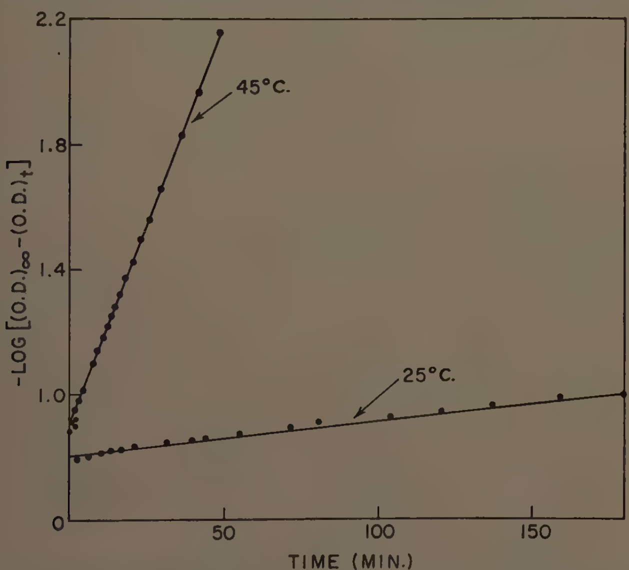

FIGURE 4. The rate of reaction of TMV-RNA with formaldehyde in 0.1 M phosphate buffer; *p*H 7, at 25° and 45° C.

as in the formaldehyde reaction, that the effect of base pairing is reflected in a substantially lowered rate of reactivity; in this case a quantitative relation is evident.

As a final example, we refer to the work of Kalnitsky *et al.*[30] in this monograph, who have observed unexpected rises in the rate of ribonuclease digestion of RNA with increasing temperature. The increase in rate over the normal expectation can be directly correlated with the increase in optical density; it therefore conforms to the view presented here.

Identification of the Hypochromicity Change with Configuration[12]

Reactivity studies strongly support the interpretation of extensive hydrogen bonding in RNA and justify the semiquantitative detection of base pairs in terms of the hypochromic effect. They leave unanswered, however, the question of whether the hydrogen-bonded base pairs are organized in small helical

regions or at random. The hypochromic shifts observed with variations in temperature arise from electron redistribution in the purine and pyrimidine rings, which results from the formation or breaking of hydrogen bonds involving these rings. Since it is possible that these changes would occur for randomly organized base pairs, as well as for those arranged in helical regions, we can only infer the presence of the latter from the character of the temperature variation. Thus, another kind of observation is needed that will be sensitive to the helical configuration rather than to hydrogen bonding, and optical rotation offers this possibility. If an RNA molecule is randomly coiled so that it is devoid of any regular structure, its optical activity will be simply that arising from its

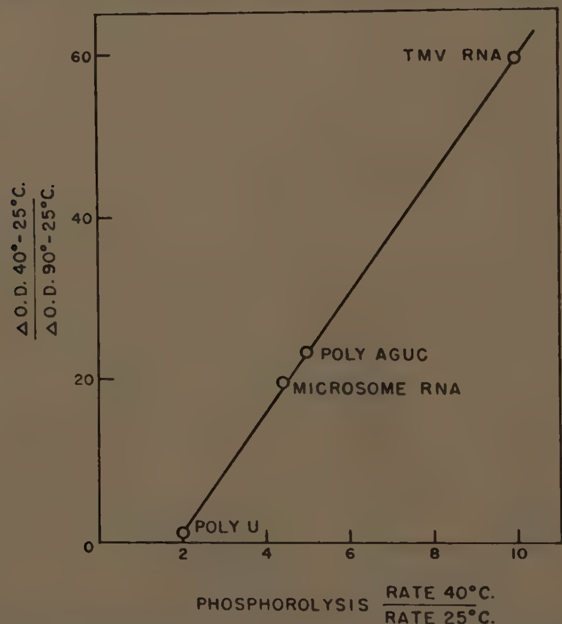

FIGURE 5. The relation between temperature dependence of the rate of phosphorolysis and of the hyperchromicity of polyribonucleotides.

ribose rings, because these are the only sites of asymmetric carbon atoms. In the helical configuration, however, a new and substantial contribution to the optical activity may arise as a result of the unique and uncompensated mutual interactions that a helical configuration of a single-handedness (right-handed for DNA and poly A + U) makes possible. This effect makes a contribution of about $-100°$ in the $[\alpha]_D$ of polypeptides in the helical configuration,[1-3] and about $-300°$ for the triple-stranded molecules of collagen.

In view of these considerations, we have investigated the optical rotation of polynucleotides and RNA as a function of temperature in a manner comparable to the optical-density studies described above. In the completely helical synthetic polyribonucleotides, the helix-coil transition would be expected to be reflected in a parallel manner for the optical density and optical rotation. The changes in both these properties should be proportional to the fraction of resi-

dues in the helical configuration since, in these complexes, there is a direct correlation between the disruption of hydrogen bonds with the melting of the helix. The test of our hypothesis of helical regions in RNA would lie then in finding if the optical density-temperature profile was congruent with the optical rotation-temperature profile, and if the magnitude of the change was reasonable when compared with the magnitude of the change for the complete helix-coil transitions in the synthetic polynucleotides.

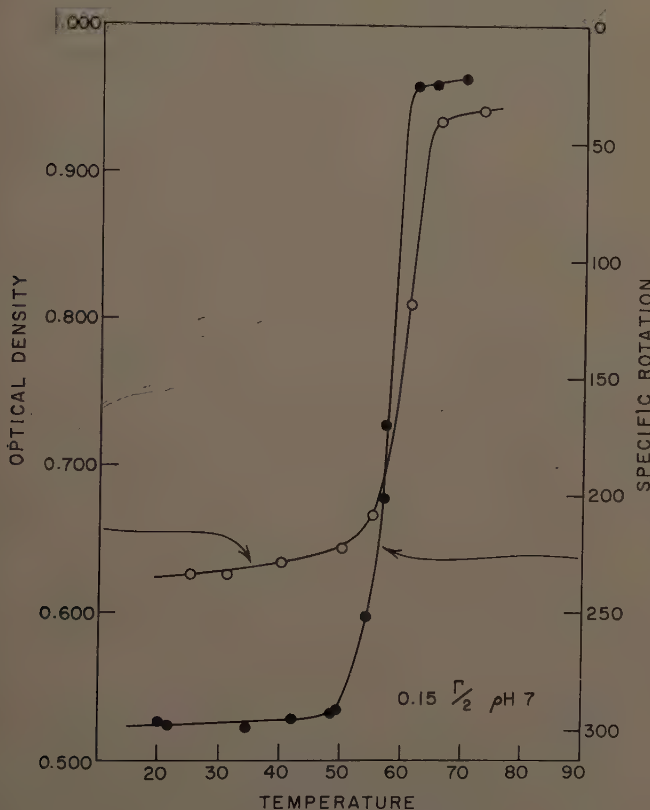

FIGURE 6. The variation in specific rotation (*filled circles*) and optical density (*open circles*) according to temperature of solutions of poly A+U.

For some time we had known that the thermally induced helix-coil transition in poly A is accompanied by a specific rotational change of about 300°.[31] Our recent results for poly A + U are shown in FIGURE 6, where it can be seen that the 51 per cent increase in optical density is matched by a congruent change in specific rotation from +300° to +25°. Since the specific rotations of the appropriate nucleosides average approximately 0° and lie within the range of +50° to −50°, the value observed for the poly A + U helix can be assumed to be close to that of completely helical RNA, a hypothetical case. The high-temperature value of +25° should not be far from that of RNA at elevated temperatures.

Thus, if the hydrogen bonds in RNA exist only in helical regions, we should expect that the optical rotation would fall from $+190°$ for TMV-RNA $[0.60 \times (300° - 25°) + 25°]$ at room temperature to about $+25°$ at the high-temperature plateau.

Prior to the specific rotation measurements on RNA, which we shall now present, Gierer[32] observed that $[\alpha]_D$ for TMV-RNA fell from $+180°$ to about $+20°$ upon exhaustive enzymatic hydrolysis. While such a change might arise from the cleavage of bonds connected to the ribose ring, its origins may well lie in the disruption of helical regions.

The critical experiment, the optical rotation-temperature profile for TMV-RNA, is shown in FIGURE 7 together with the optical density-temperature profile taken from FIGURE 3. It is clear that the 2 profiles can be brought into coincidence by adjusting the ordinate scales. Moreover, the low- and high-

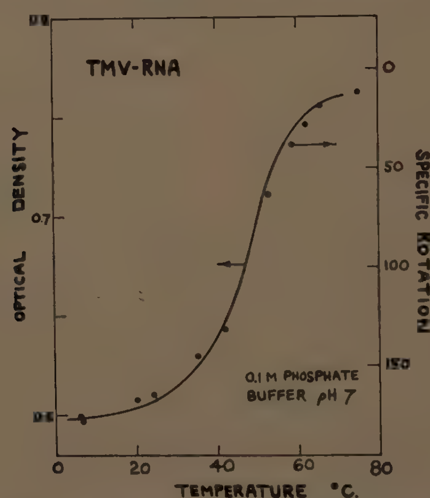

FIGURE 7. The variation in specific rotation (*open circles*) and optical density (*solid line*) according to temperature of solutions of TMV-RNA.

temperature limiting rotations are essentially those predicted from poly A + U. From this, we conclude that the changes in the optical density of RNA are due directly to the formation and rupture of hydrogen-bonded base pairs that are organized in helical regions.

Having reached this conclusion, it is necessary to examine as carefully as possible the ways in which it can be satisfied structurally. Specific ways must be found in which as much as 40 per cent of the residues in a single polynucleotide chain of random sequences can be brought into helical regions (the 60 per cent estimated for TMV-RNA may arise in part from favorable specific sequences). A proper study of this problem has not been made, but the following points appear relevant.

Since the predominant two-stranded helical polynucleotides are antiparallel, this condition may be assumed with some confidence for the helical regions in question. This suggests that the helical regions consist of two chains running in opposite directions. As the solution is cooled and these regions form, it is

likely that neighboring sections of the chain can explore each other most readily to see if a stable union can be made. Consequently, it would be expected that most of the regions would consist of hairpinlike sections of the chain with a small but variable number of bases unmatched in the loop. The quest for the most stable configurations is, of course, simply an exhibition of the intense internal Brownian motion in which all flexible parts of the chain participate. However, it is facilitated by the relatively great flexibility of polynucleotide chains and the intrinsic ability they have for lateral motion with respect to one another. This latter point is evident from the way in which essentially all base pairs are formed when poly A and poly U (and other pairs that form helical complexes) are mixed.[8] This efficiency clearly involves the lateral motions of one chain with respect to the other until all the gaps in the helical structure are filled.

A second feature that may be anticipated in these helical structures is the inclusion of unmatched bases—that is, defects. We have not yet explored all base pairs that might be hydrogen-bonded in neutral saline solution. However, it appears that opposing pyrimidines would be most easily accommodated for this and would involve no steric hindrance. Certain purine-pyrimidine pairs (G-U and A-C) might be unmatched and would involve some distortion, but they appear to be acceptable in small amounts. Purine pairs are most likely unacceptable; consequently, their occurrence would terminate a helical region.

The other determinative feature of the problem is the limitation imposed by the minimum helix length needed for stability. This is a problem that can be solved in time for helices not containing defects. This, as well as the estimate of the extent to which defects can be incorporated, calls for considerable experimental work before quantitative discussion is possible. However, by outlining the problem in this way it should be clear that we are not faced with the simple statistical problem of what fraction of residues could be accommodated in DNA-like regions of a given length, assuming random sequence in the chain. This, of course, would lead to a much smaller figure than do the estimates in the preceding sections. However, the additional aspects of the structural problem described here appear to provide sufficient latitude to make our estimation of the fraction of nucleotides in helical regions tentatively acceptable.

The only other observations that bear directly on our conclusions appear to be those of Rich and Watson,[33] who carried out a comparative X-ray examination of DNA and RNA. The RNA was poorly orientable, but weak, diffuse reflections characteristic of the DNA X-ray pattern were obtained. Despite the low quality of the RNA diffraction pattern, it was concluded that it had no discernible reflections in the meridional regions where helix-diffraction theory precludes the occurrence of reflections. Thus it appears that the X-ray diffraction of RNA is consistent with the short, imperfect, and poorly orientable helices proposed by us. However, the quality of the RNA diffraction is such that the lack of conflict is all that can be properly claimed.

It is of interest to observe that the secondary structure of RNA proposed here is formally very similar to that being increasingly adopted for globular proteins. Considerable evidence is now available indicating that specific fractions of the amino acid residues are in helical regions.[1, 3, 34] At usual ionic strengths (0.01 to 0.1), RNA molecules are swollen roughly tenfold with water and are, there-

fore, much less rigid than globular proteins. This partially swollen state probably prevents display of a recognizable tertiary structure such as is exhibited by proteins. However, it is perhaps worth emphasizing the fact that the secondary structure—that is, the helical regions—in RNA is much more stable intrinsically than that in proteins. This is seen from the fact that all proteins denature at much lower charge densities than that always present on RNA at neutral *p*H—that is, 1 charge per residue.

Ribonucleic Acid in Microsomal Particles

Since RNA occurs principally in close association with protein in microsomal particles and fulfills its biological function there, it is natural to inquire if the methods and concepts developed above can be applied to elucidate the configuration of RNA in microsomal particles. A preliminary investigation of this has been made involving the thermal dependence of hypochromicity and formaldehyde reactivity.[26]

Microsomal particles were prepared from calf liver and purified to the extent that at least 90 per cent of the sample had a sedimentation coefficient of 81 *S*. The intrinsic viscosity in the same solvent, 0.02 M phosphate buffer, was 0.039. From the Einstein-Stokes equation these results may be used to calculate a molecular weight of 3,700,000. This indicates that the spherical particles in solution are 200 Å in diameter and contain equal volumes of water. The diameters of 100 to 150 Å obtained with the electron microscope[35] are consistent with our observations, since the water will have been removed and the particles shrunken in the process of preparing them for electron microscopy.

Upon measuring the optical density as a function of temperature it was found that no change occurred in the 0.02 M buffer until about 55° C. At higher temperatures the optical density rose, but the complete profile could not be obtained in the available temperature range. This became possible, however, when the buffer was reduced to 0.001 M phosphate; the results are shown in FIGURE 8. In this same solvent RNA itself would have a profile lying at considerably lower temperatures. The comparison at the same ionic strength is not warranted, however, because of the much greater net charge on the RNA and the lowering of the melting out that this causes. At the higher ionic strength of 0.02 M phosphate the optical density-temperature profile is moved to higher temperatures so that its upper part nearly coincides with that of the microsomal particles. This result is included in FIGURE 8. Comparison shows that the first third of the transition that occurs in RNA is missing in the microsomal particles, but the remainder coincides. This preliminary experiment can be tentatively interpreted as indicating that the helical content of RNA in the microsomal particles is only two thirds as much as in isolated RNA, but that the additional helical regions in isolated RNA are particularly weak; they may indeed arise in a nonspecific way after removal of the protein.

Optical-rotation studies have not yet been made on the particles; consequently, this tentative interpretation is not as strongly supported as the earlier work already described. In particular, we should know, before the optical density changes can be clearly interpreted, if the interaction of protein with RNA can affect its ultraviolet absorption.

Nevertheless, equally preliminary studies of formaldehyde reactivity do sup-

port our tentative conclusions. In these, it was observed that, in 0.001 M buffer, a temperature of 80° C. was required to bring about complete reaction in the particles in 10 hours, whereas a temperature of only 55° C. was required for RNA. When the reactivity was measured as a function of temperature, a marked increase was found to set in at about 55° C. for the particles. Thus, we have 2 indications of base pairing and helical arrangement in microsomal particles. Specifically, these show that about 25 per cent of the nucleotides are in helical regions and that these are not subject to thermal disruption at less than 50° C. in 0.001 M salt, or at higher temperatures at higher ionic

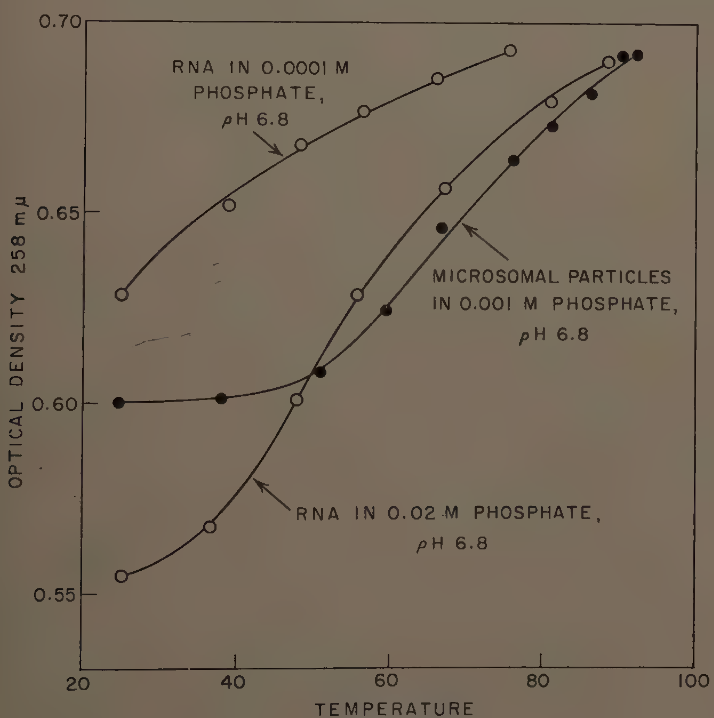

FIGURE 8. The variation in optical density according to temperature of solutions of RNA and microsomal particles.

strengths. If these tentative conclusions prove true, it would appear that the helical regions are an important aspect of the molecular architecture of microsomal particles, and that their functional role deserves investigation.

Acknowledgments

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AN ANALYSIS OF THE RELATION BETWEEN DNA AND RNA

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Introduction

The purpose of this paper is to discuss the relationships that may exist between the sequence of nucleotides in desoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It is concerned with determining which kinds of relationships are possible and which unlikely, and not with deciding upon a particular mechanism.

A great deal of biochemical work currently is directed toward understanding various aspects of the function, metabolism, and structure of both RNA and DNA. The importance of nucleic acids stems from our belief that they have a central directive role in guiding the development of a biochemical system and in maintaining many of its characteristic features.

Since the classic experiments of Avery (1944), done more than 20 years ago, we have known that the DNA molecule influences cellular activity profoundly. Avery's transforming experiments showed that the DNA from one strain of bacteria not only could alter the metabolic characteristics of another strain, but that this alteration was inherited by all subsequent progeny. The genetic role of DNA was again clearly demonstrated by Hersey and Chase (1952) when they showed that only the DNA from the bacteriophage virus was injected into the bacterial host, carrying with it all the genetic information present in the invading virus. If 2 different strains of bacteriophage viruses infect the bacterial host cell, genetic recombination can be demonstrated between the 2 invading types of viral DNA.

Recently the biochemical role of RNA has been clarified to a limited extent. RNA is found in the cell in 2 quite different forms, most of it being closely bound to protein in the microsomal particles present in the cellular cytoplasm. These particles have a molecular weight near 4 million, and the RNA component accounts for approximately one half of the particle mass. The remaining RNA in the cytoplasm is called "soluble RNA" because the molecules do not sediment in the high centrifugal fields used to precipitate the microsomal particles. A great deal of attention has been directed recently to the role of the microsomal particles in protein synthesis. Thus, it has been demonstrated that this is the site where activated amino acids are organized into the specific sequences necessary to manufacture protein molecules (Littlefield *et al.*, 1955). Recent work on the protein component of microsomal particles has shown that the amino acid composition is very similar in particles obtained from a variety of sources such as rat liver, pea seedlings, or mammalian reticulocytes (Ts'o *et al.*, 1958). These observations have reinforced the belief that the sequence of amino acids in protein synthesis is determined somehow by the sequence of the nucleotides in the RNA component of the microsomal particle. This point of view perhaps may best be regarded as a useful hypothesis on the basis of which we can frame experiments. Many attempts have been made to describe systems of polynucleotide-amino acid

interaction that would account for the specific sequence of amino acids observed in proteins (Gamow *et al.*, 1956; Crick *et al.*, 1957). Although most of the experiments carried out with microsomal particles are compatible with the concept that the RNA nucleotide sequence determines the amino acid sequence, we do not have sufficient information at present to regard it as other than an attractive and plausible hypothesis.

It is most interesting that another fundamental function can be demonstrated for the RNA molecule: namely, its ability to act as a carrier of genetic information. This is seen most clearly in experiments with the purified RNA of the tobacco mosaic virus. The tobacco plants can be infected by viral RNA in the absence of the protein coat normally found on the rod-shaped virus (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956). Once the RNA has invaded the leaf cell, it is able to produce more virus particles that contain all of the genetic information present in the original virus particle. The infectivity of viral RNA has also been demonstrated with other RNA viruses (Colter *et al.*, 1957; Ada and Anderson, 1959).

Is DNA Related to RNA?

With the exception of the case of the RNA viruses cited above, it is widely held that genetic information is carried entirely by DNA. When we use the phase "genetic information," we are describing a process whereby substances inherited from parent cells influence the functional characteristics of daughter cells. Genetic control on the biochemical level is believed to be mediated almost entirely through the development of the enzymes that catalyze specific biochemical reactions and that therefore determine the metabolic characteristics of the developing and adult organism. Thus, genetic information must control protein synthesis. These views have led to a hypothesis that holds that there must be a relationship between the DNA that carries genetic information and the RNA molecules that apparently are active in the final protein synthetic reaction. It should be noted that the genetic DNA need not be related to all the cellular RNA but, rather, only to that part of it in the microsomal particle that is active in determining the sequence of amino acids. It is not at all unlikely that some RNA is unrelated to DNA: for example, the soluble RNA. It is often stated that DNA "makes" RNA, and many now regard this as almost axiomatic. It should be emphasized strongly that there is no clear proof for this view; it is the logic of the analysis rather than the weight of experimental evidence that makes us believe that there must be a relation between the nucleotide sequence of the two nucleic acids. However, other alternatives cannot be ruled out unambiguously. At one time it was thought that DNA might play a minor but nonetheless direct role in stimulating specific protein synthesis in cell nuclei. This view recently has been discredited, but still exists as a possibility (Allfrey and Mirsky, 1958).

Various mechanisms for relating the base sequence in DNA to that in RNA are listed in TABLE 1. The first category listed is that in which there is no relation between the nucleotide sequence in DNA and that in RNA. We may rule this out as unlikely in light of the discussion above.

A second possibility is that in which the sequence of bases in RNA is deter-

mined by both protein and DNA. This implies a substantive role by protein in at least partly determining the sequence of bases in RNA, and not a role in which the protein is confined to a specific catalytic action, such as is found in an enzyme. All mechanisms considered for specific RNA synthesis involve the participation of various enzymes. While the latter are an integral part of a synthetic reaction, they may not necessarily be active in the determination of nucleotide sequence. If, however, they are important in nucleotide sequence determination, then we should be able to relate a certain sequence of amino acids plus a certain DNA nucleotide sequence to a particular sequence of RNA nucleotides.

There are several reasons why a mechanism of this type is less probable, even though we cannot rule it out of consideration entirely. In a system of this type we must imagine a systematic interaction between a growing ribonucleotide chain and another templatelike structure composed of DNA and protein.

TABLE 1

MECHANISMS THAT MAY RELATE DNA BASE SEQUENCE TO RNA* BASE SEQUENCE

(I) There is no relation between the two nucleic acids and, hence, no mechanism.
(II) DNA base sequence plus an amino acid sequence in proteins defines the RNA base sequence.
(III) DNA base sequence alone determines the RNA base sequence.
- (*A*) Other molecules act as intermediates between the sequences of DNA and RNA nucleotides.
- (*B*) Two-stranded DNA molecules act as template sites.
  - (1) The template is a "native" DNA molecule.
  - (2) The template is a helical DNA molecule with one of the hydrogen bonds between the base pairs broken. The DNA is in an "activated" state.
- (*C*) A single-stranded DNA molecule provides template sites.

* The RNA referred to is the RNA of the microsomal particle, which may have an important role in determining the sequence of amino acids.

In this structure, the DNA and the protein must both interact systematically with the ribonucleotide chain in such a way that the choice of polymerizing a given purine or pyrimidine base is determined substantially by the ordering of the desoxynucleotides and amino acids. Despite considerable effort in this direction, it has not been possible to derive a model of such a system whereby the amino acid side chains could interact systematically with the purine and pyrimidine bases in a selective fashion.

Another way of looking at this problem is to consider which features of the RNA side chains, that is, the purines and pyrimidines, give rise to selectivity. Roughly speaking, the four purine and pyrimidine residues are quite similar. They are flat heterocyclic molecules with an unsaturated π-electron system, and they have amino and keto groups attached to the rings. In sharp contrast to this, the amino acid side chains are quite varied chemically. They include nonpolar aliphatic groups, aliphatic groups with electronegative substituents that can be positively or negatively charged and, finally, unsaturated ring systems. It is difficult to imagine a systematic chemical interaction that would produce nucleotide sequence selectivity. Accordingly, we must regard this as an unlikely mechanism, although perhaps not an impossibility.

DNA as a Template

The DNA molecule could act as a template for determining RNA nucleotide sequence by organizing a sequence of residues in another molecule that could act as an intermediate between the two: for example, a protein or an amino sugar polysaccharide. The arguments against such a mechanism are of the same type as cited above for the exclusion of protein in determining nucleotide sequence. We cannot be certain that this type of mechanism does not occur, even though it is regarded as highly improbable.

The most characteristic feature of the nucleic acids is the repeating array of purine and pyrimidine bases, with their attached amino groups and keto groups that can form a specific pattern of hydrogen bonding. The mechanisms listed in TABLE 1 under III *B* and III *C* are dependent upon a systematic interaction between polydesoxynucleotide chains and polyribonucleotide chains. All of these interactions are mediated through a highly specific pattern of hydrogen bonds formed between the purine and pyrimidine residues. These are specific because of the directionality of the hydrogen bond and because it has a characteristic length that is a function of the particular electronegative atoms bound by the proton (Donohue, 1952); these result in considerable stereochemical specificity.

The first mechanism to be considered is that in which the template for organizing oncoming ribonucleotide residues is the native DNA molecule itself. In such a reaction we imagine a polyribonucleotide chain growing in the helical groove of the DNA molecule, each oncoming ribonucleotide base forming hydrogen bonds to the 2-stranded DNA molecule. The DNA molecule has 4 types of sites on it relative to the direction of the growing ribonucleotide chain; these are shown diagrammatically in FIGURE 1. The 4 sites arise out of the 2 base pairs, each of which may be oriented in 2 directions relative to the oncoming RNA chain, which has a directionality: that is, the sense of the O_3'—O_5' internucleotide ester linkages. A purine or pyrimidine base of the RNA molecule would form a different set of hydrogen bonds to the adenine-thymine base pair of DNA if the adenine residue were on the left in one case or on the right in the other, since the ribonucleotide base cannot rotate about its glycosidic bond.

The first attempt to work out a systematic interaction of this type by using a model of the DNA molecule as a template was made by Orgel and Watson in 1954 (personal communication). The problem they considered was that of orienting a ribonucleotide base in the wide groove of the DNA molecule so that it was held in place by 2 hydrogen bonds, 1 to each base of the DNA base pair. Thus, there is 1 residue per 3.4 Å along the helical axis. The basic problem is that of finding a model in which all the glycosidic bonds in the ribonucleotide chain occur in the same position and orientation relative to the axis of the DNA molecule; this is illustrated schematically in FIGURE 2*a*. If this can be accomplished, then the native DNA could act as a template site for polymerizing an RNA chain with a specific base sequence. Orgel and Watson found an arrangement they considered interesting, but they were not convinced that it was quite feasible stereochemically. A scheme similar to this has been illustrated in a review article by Stent (1958), in which he shows a

system for hydrogen-bonding a third ribonucleotide base onto the DNA base pairs. In his system the ribose adenine (A) residue is bound to the thymine-adenine pair, the uracil (U) residue is bound to the adenine-thymine pair;

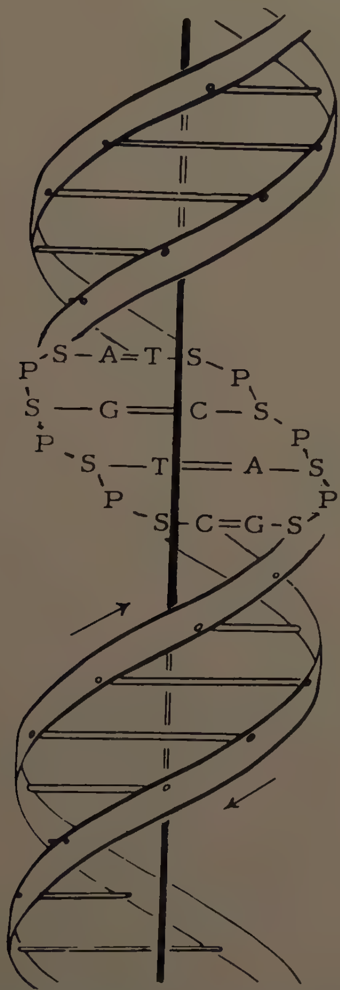

FIGURE 1. Diagram illustrating the four types of sites present in the groove of the DNA molecule. *S* represents desoxyribose sugar; *P*, the phosphate group; *A*, adenine; *T*, thymine (5 methyl uracil); *G*, guanine; and *C*, cytosine.

similarly, guanine (G) is hydrogen-bonded to the cytosine-guanine pair and cytosine (C) to the guanine-cytosine pair. However, this arrangement is not stereochemically correct because the hydrogen bond angles must be distorted by various amounts in order to form the 2 hydrogen bonds necessary to link the oncoming ribonucleotide base. In addition to this, an unfavorable tautomeric shift is postulated on the oxygen group of the guanine residue.

It may be possible to obtain experimental evidence that gives some support to a scheme of this sort, since it predicts that a DNA with a high $\frac{A+T}{G+C}$ ratio would produce an RNA with more A and U than G and C. Thus, Belozersky and Spirin (1958) have analyzed the composition of the DNA and RNA in a variety of bacterial nucleic acids. They have been able to show that there is a rough but nonetheless observable correlation between the ratio of $\frac{G+C}{A+T}$

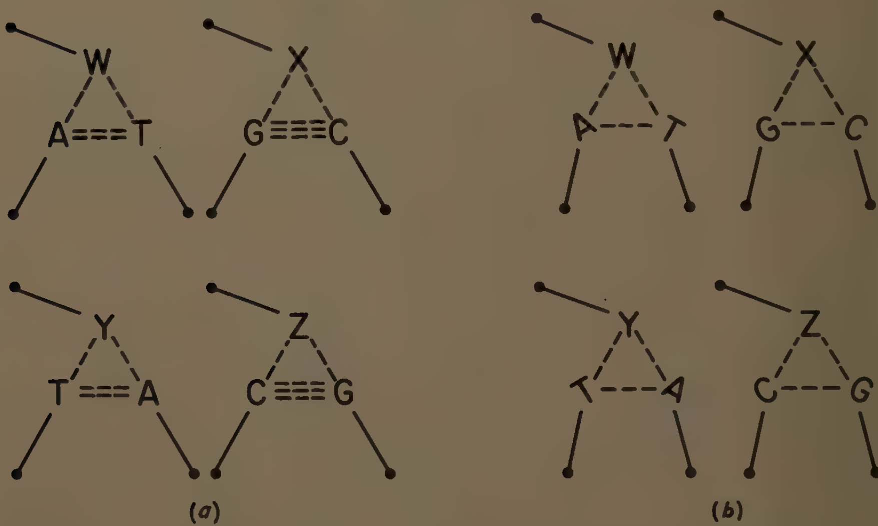

FIGURE 2. Examples of possible types of hydrogen-bonding of ribonucleotide bases *W*, *X*, *Y*, and *Z* to the DNA base pairs. Hydrogen bonds are represented by dashed lines, glycosidic bonds are represented by a solid line, and the dot represents the sugar-phosphate chain. (*a*) Hydrogen-bonding to the DNA base pairs held together by an intact set of hydrogen bonds. (*b*) Cyclic hydrogen-bonding involving one ribonucleotide base and two DNA bases held together by only one hydrogen bond.

in DNA to the ratio of $\frac{G+C}{A+U}$ in RNA. This would suggest that a mechanism in which the adenine-thymine base pair coded for the adenine and uracil bases of RNA might be occurring in this system. However, experimentally the relationship is only a very approximate one. Furthermore, opposite conclusions can be drawn from a study of the base ratios of the nuclear DNA and nuclear RNA in several nonbacterial sources (Vincent, 1952; Elson and Chargaff, 1955). In these cases, high ratios of DNA $\frac{A+T}{G+C}$ are associated with high ratios of $\frac{G+C}{A+U}$ in the nuclear RNA. Thus, it is by no means clear that analytical data of this type can be used to support a particular coding mechanism.

Experimental evidence for the existence of 3-stranded polynucleotide com-

plexes has been obtained in studies with the synthetic polyribonucleotides. These experiments have a bearing on the relationship between DNA and RNA, since it has been possible to demonstrate that the combination of polyadenylic acid plus polyuridylic acid results in a 2-stranded helical molecule similar, in many respects, to DNA (Rich and Davies, 1956). X-ray diffraction studies show that the 2 polynucleotide chains are helically coiled about each other with the bases hydrogen-bonded through the same hydrogen bonds that hold together the adenine-thymine base pair in DNA. It has been found experimentally that this 2-stranded polynucleotide helix is capable of taking on a third polynucleotide strand in a specific fashion (Felsenfeld *et al.*, 1957). Thus the polyadenylic acid plus polyuridylic acid complex will hydrogen-bond with a third strand of polyuridylic acid, but will not hydrogen-bond with a third strand of polycytidylic acid or polyadenylic acid. These experiments show that it is possible for 3-stranded polynucleotide complexes to form, and we can use this system to study their stability. However, for this 3-stranded polynucleotide structure to be an example of the type of a DNA-RNA complex discussed above, the hydrogen bonds between the third strand of polyuridylic acid and the original 2-stranded complex should be of the type shown in FIGURE 2*a*, with the uracil of the third strand in contact with both bases of the initial 2-stranded structure. However, an X-ray diffraction study of the molecular structure of the 3-stranded complex of polyadenylic acid plus 2 polyuridylic acid strongly suggests that the third chain of polyuridylic acid is hydrogen-bonded to the polyadenylic strand alone through the amino group of adenine and nitrogen 7 of the imidazole ring (Davies and Rich, unpublished observations). In addition, the fact that the polyadenylic acid plus polyuridylic acid complex does not bind a third strand of polyadenylic acid suggests that a mechanism of the type described above may not occur with the DNA molecule itself. In an attempt to assess the importance of the methyl group found on the thymine residue of DNA, polyribothymidylic acid was synthesized (Griffin *et al.*, 1958). This molecule also combines with polyadenylic acid and is a closer analogue of DNA. However, this 2-stranded helical complex of polyadenylic acid and polyribothymidylic acid will combine with polyuridylic acid, but not with a third strand of polyadenylic acid. Thus, we have both positive and negative evidence regarding the possible synthesis of an RNA molecule in the groove of a native 2-stranded DNA molecule. Further experiments will be needed to evaluate fully a mechanism of this type.

Another possible mechanism involving a 2-stranded DNA molecule is illustrated diagrammatically in FIGURE 2*b*. The DNA molecule is still helical, but the configuration is altered to an "activated" state. The base pairs of DNA are now hydrogen-bonded by only one bond, as shown in the diagram; the other hydrogen bonds have been broken by a rotation of the planar purine and pyrimidine bases. The DNA bases are now able to form hydrogen bonds with a third ribonucleotide base (W, X, Y, or Z), as shown in FIGURE 2*b*. This is an example of cyclic hydrogen-bonding in which each of the 3 purine or pyrimidine residues is bonded to the 2 neighbors by single hydrogen bonds. A mechanism of this type was suggested by the finding that polyinosinic acid has the form of a 3-stranded helical molecule with the purine bases hydrogen-bonded in the center of the molecule, as shown in FIGURE 3 (Rich, 1958).

Models were built in an attempt to organize a cyclically hydrogen-bonded structure with 2 DNA chains and 1 RNA chain. However, despite vigorous efforts in this direction, no convincing specific model of triplets of bases could be made in this manner.

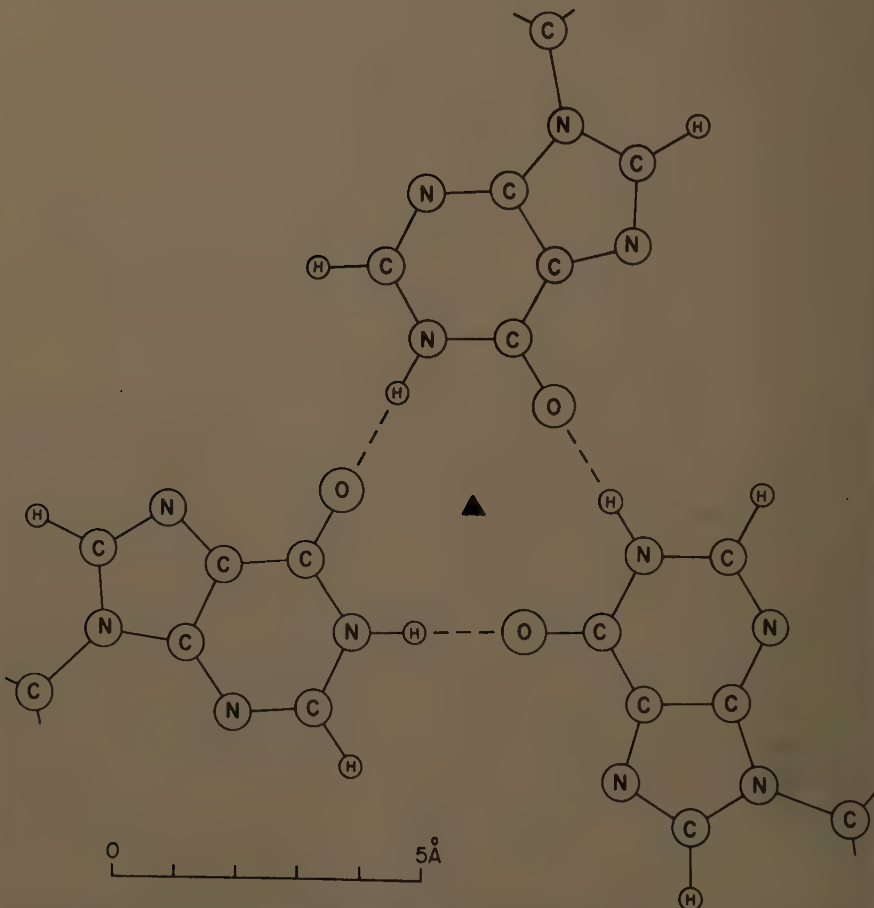

FIGURE 3. The hydrogen-bond system found in polyinosinic acid. The purines are hydrogen-bonded to each other around a threefold symmetry axis.

An interesting attempt recently has been made by Zubay (1958) to construct a model of this sort in which the DNA bases are not rotated in the plane at right angles to the helical axis, but instead are tilted in an unusual manner, so that a system of cyclic hydrogen bonds is organized between the two DNA bases and the third ribonucleotide base. There are two disadvantages to Zubay's proposal: (1) the scheme lacks specificity, as he points out, since there are two possible systems of hydrogen-bonding the three bases in this tilted form, and each would produce a different RNA molecule; (2) the amino groups of adenine are rotated out of the plane of the adenine residue, and this results

in an amino hydrogen atom being buried in the center of the triplet of bases without hydrogen-bonding to any electronegative atom; such a systematic arrangement would produce considerable instability in the three-stranded complex.

We can summarize the efforts made toward finding a template site on a two-stranded DNA molecule by saying that, although many attempts have been made, there has been no convincing demonstration that such a mechanism is stereochemically feasible. While these failures certainly do not rule out the possibility of a two-stranded DNA template, it has stimulated thinking about the possibility of a single-stranded DNA acting as a template for the manufacture of RNA.

Single-Stranded DNA Templates

Before discussing single-strand mechanisms for producing RNA, it is worth considering the information we now have, through the work of Kornberg and his associates, about the mode of replication of DNA (Lehman *et al.*, 1958). It is possible to isolate an enzyme (DNA polymerase) that utilizes the four desoxynucleotide triphosphates and a primer of DNA to produce new molecules of DNA that have the same nucleotide composition and, therefore, probably the same nucleotide sequence as the original primer molecule. Usually, in the absence of primer DNA, no reaction occurs. The rate of polymerization of new DNA is increased by thermally denaturating the primer DNA, a denaturation process in which the DNA strands are partially separated but not hydrolyzed. It has been found that the best primer for this enzyme is a DNA obtained from the small virus ØX-174, believed to be a single-stranded molecule (Sinsheimer, 1959). The product of the reaction primed by the single-stranded DNA appears to be the usual double-stranded DNA molecule. Thus, these experiments strongly suggest that the enzymatic production of new DNA molecules is carried out by a single-stranded DNA chain that probably acts as a template for the oncoming desoxynucleotides through the specific hydrogen-bonding that is seen in the DNA molecule. These hydrogen bonds apparently have considerable selectivity, since the composition of the newly synthesized DNA remains constant even though the ratio of the individual desoxynucleotide substrates is varied over a wide range (Lehman *et al.*, 1958). One can describe a DNA replication cycle (FIGURE 4); it has 2 phases, in one of which the molecule exists as a double-stranded helix, as shown on the right side of the diagram. The 2-stranded molecule undergoes chain separation to produce 2 single-stranded molecules. The single-stranded form usually has only a transient existence in most organisms, and probably exists as a single strand over only a small segment of the molecule. Indeed, at the present, it is only in this very small virus that the single-stranded DNA molecule has been seen in a natural system. In the presence of the appropriate enzyme and activated nucleotide residues this single chain acts as a template for chain replication; the result of this polymerization is a 2-stranded molecule, as shown on the right side of the diagram.

It is reasonable to ask whether this mechanism for DNA replication may be an analogy for the mode of production of RNA. The last mechanism listed

in TABLE 1 is one in which a single-stranded DNA molecule acts as a template for the production of an RNA molecule by a process that is analogous to that described above for the production of a new single strand of DNA. In order to consider the feasibility of this type of template mechanism we must ask whether it is stereochemically possible to have a hybrid 2-stranded helical molecule with DNA on 1 strand and RNA on the other. Could these 2 chains be united by the same types of systematic hydrogen bonds found in the DNA molecule itself? We can attempt an answer to this question by surveying the results of molecular-structure studies on the synthetic polyribonucleotides and on DNA itself. The substitution of uracil for thymine does not effect the hydrogen-bonding. We must now consider whether the additional hydroxyl group on the RNA backbone may hinder the formation of this kind of structure.

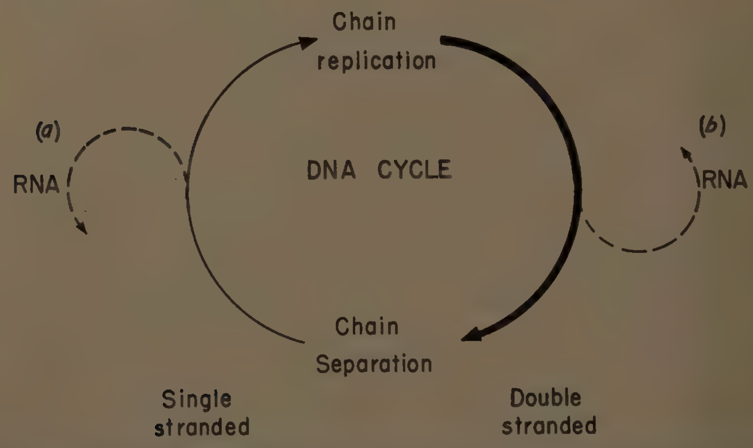

FIGURE 4. Diagram illustrating the DNA reproduction cycle. The two alternatives for RNA production from DNA are: (*a*) a single-stranded DNA template, or (*b*) a double-stranded DNA template.

As mentioned above, the synthetic polyribonucleotides, polyadenylic acid and polyuridylic acid, combine to form a 2-stranded helical molecule with the adenine and uracil residues hydrogen-bonded. Most pertinent, however, is the fact that the helical screw of this 2-stranded complex is identical to that seen in the DNA molecule—that is, a translation of 3.4 Å (the thickness of the purine-pyrimidine base pair) and a rotation of 36° (Rich, 1957). The only difference between these 2 helical molecules is the fact that the radius of the synthetic helix is somewhat larger than that seen in DNA, undoubtedly due to the additional oxygen on the backbone. However, it seems quite likely, on stereochemical grounds, that a single-stranded RNA molecule could be wrapped around a single-stranded DNA molecule with complementary hydrogen-bonding. In this hybrid DNA-RNA molecule, the helical axis would be slightly displaced from the position it occupies in DNA. It should prove possible to resolve this point experimentally by using synthetic polynucleotides to form such hybrid molecules.

The advantage of this type of replication mechanism is that it utilizes a

series of specific complementary hydrogen bonds that we know to be of high accuracy in the replicating process, since they are the same interactions that are undoubtedly responsible for the replication of DNA. In summary, since the individual residues of DNA and RNA are very similar stereochemically, it is not unreasonable to believe that nature may use a fundamentally similar mechanism for replicating either molecule.

We have ignored one type of ambiguity in many of the replication mechanisms discussed above. This arises from the fact that DNA is two-stranded, with the backbone chains running in antiparallel fashion. If the two-stranded molecule is used as a template, the growing RNA chain, by virtue of symmetry, could start from either end of the molecule. It would then be necessary to postulate that one end of the DNA molecule is differentiated in some

TABLE 2
POSSIBLE TYPES OF NUCLEOTIDE POLYMERASE ENZYMES

Enzyme	Primer	Substrate	Product
A	Single-chain DNA	Desoxy ATP Desoxy GTP Dexoxy CTP Desoxy TTP	DNA polynucleotide chain
B	Single-chain DNA	ATP (ADP) GTP (GDP) CTP (CDP) UTP (UDP)	RNA polynucleotide chain
C	Single-chain RNA	ATP (ADP) GTP (GDP) CTP (CDP) UTP (UDP)	RNA polynucleotide chain
D	Single-chain RNA	Desoxy ATP Desoxy GTP Desoxy CTP Desoxy TTP	DNA polynucleotide chain

way so that RNA replication could start there instead of at the other end. If a single-stranded molecule is a template, we remain with an ambiguity arising from the fact that the DNA molecule has two complementary halves. Either of these can be a template for the DNA polymerase enzyme. If we were polymerizing RNA molecules on single-stranded DNA, we should be making two classes of complementary RNA molecules from the two complementary DNA strands. This will be true unless there is a mechanism for suppressing the polymerization of one of them.

Types of Single-Stranded Template Mechanisms

We have seen that it appears to be stereochemically reasonable to think of combining a single RNA chain with a single DNA chain to form a two-stranded complementary helix similar to that seen in the DNA molecule. If this were a generalized mechanism for reproducing polynucleotide chains, other similar types of reactions could occur. In TABLE 2 are listed the four types of nucleo-

tide polymerase enzymes that are possible for the replication of polynucleotide chains. All of these are presumed to act by polymerizing new polynucleotide strands, using a given single-chain polynucleotide as a primer.

Enzyme *A* of TABLE 2 catalyzes a reaction in which a single-chain DNA primer utilizes the 4 desoxynucleotide substrates to produce a new DNA polynucleotide chain with a sequence complementary to that of the primer. This is, of course, the DNA polymerase enzyme that Kornberg and his associates have isolated.

Enzyme *B* of TABLE 2 uses a single-chain DNA molecule as a primer, but has as substrates the ribonucleotide di- or triphosphates; it polymerizes a new RNA polynucleotide chain. This is the mechanism described above as being a possible means for transferring sequence information from DNA to RNA. We should consider at this point the possibility of utilizing the polynucleotide phosphorylase enzyme to carry out this kind of an RNA polymerization (Grunberg-Manago and Ochoa, 1955). One of the most interesting characteristics of polynucleotide phosphorylase is that initially it appeared to act in the absence of primer. However, on further purification it was possible to show that primer molecules were necessary in the form of small polyribonucleotides that were incorporated into the growing polynucleotide chain (Singer *et al.*, 1957). At present, there is no evidence that DNA can act as a primer for the polynucleotide phosphorylase enzyme; hence it is difficult to understand how it could be used in a system for transfering sequence information from DNA to RNA. However, it is entirely possible that the polynucleotide phosphorylase enzyme, as isolated, has been separated from other constituents present in the intact cell; this may produce primer type specificity of the kind seen in the DNA polymerase reactions. Many enzymes are believed to be organized into aggregates *in vivo* instead of existing in the form of the free-floating protein molecules that we observe upon isolation. Thus it may be possible that polynucleotide phosphorylase represents a fragment of an enzyme complex that has lost its primer specificity. Another alternative, of course, is that it operates in the cell to produce a nonspecific type of RNA that feeds the pool of soluble RNA.

In TABLE 2, *C* and *D* are the remaining two types of nucleotide polymerase enzymes that could utilize a single-chain RNA as a primer and produce either an RNA polynucleotide chain (*C*) or a DNA polynucleotide chain (*D*). At first, one is tempted to say that there is no evidence that suggests that either of these types of polymerizing enzymes exist. However, we know that the RNA component of tobacco mosaic virus, as well as of other RNA viruses, has the ability to carry genetic information. This information, residing in the nucleotide sequence, must be passed on to other molecules in order to develop a system for replicating more viral RNA as well as more viral protein. Evidence has been presented that strongly suggests that the RNA in the tobacco mosaic virus is single-stranded (Franklin, 1956; Ginoza, 1958); hence, it is not unlikely that this single strand of RNA may serve as a template for manufacturing more RNA, which may, in turn, facilitate the synthesis of viral protein. This is the system described in TABLE 2, *C*. A second possibility (TABLE 2, *D*) is one in which the single-chain RNA acts to produce a single-stranded DNA polynucleotide chain that then functions to produce more RNA and,

consequently, more protein of the viral type. This reaction seems the least likely of those listed in TABLE 2 but, as we have no experimental information, it cannot be discounted entirely.

Evolution of the Nucleic Acids

One of the really remarkable things about the nucleic acids is that there are two of them. These molecules are very similar, differing only by a systematic hydroxyl group and an occasional methyl group. Nevertheless, we see that they appear to have quite different functions in the cell. The DNA molecule is used in nature as the major carrier of genetic information; the RNA molecule seems to be used in the conversion of genetic information into actual protein molecules that carry out, as it were, the functions implicit in the genetic material.

Nothing is known about the survival of the nucleic acids in the evolutionary process. However, because we see these two closely related molecules with different functions, we are, of course, tempted to ask whether they may have originated historically from a common molecule that then specialized in the course of evolution into the two different classes of molecules we see today. To pursue this argument further, we note the fact that the RNA molecule is also able to carry genetic information, as mentioned above in the case of the tobacco mosaic virus infection. Hence it may be reasonable to speculate that the first polynucleotide molecule that nature used was an RNAlike molecule that is able both to convey genetic information and to organize the amino acid molecules to produce specific types of proteins. DNA might then be regarded as a specialized derivative molecule that evolved in a form that only carried out the molecular replicating cycle that is an inherent part of the transmission of genetic information. DNA is less reactive metabolically, perhaps because of the absence of the hydroxy group, and this may have a selective advantage in an evolving biochemical system.

Insofar as we know, all species of living material have DNA in them. If a primitive organism were found that contained RNA and no DNA, then it is conceivable that such an organism would represent a residual form from an earlier system in which the RNA molecule carried out both the genetic-carrying and protein-synthesizing functions implicit in the nucleic acids. Perhaps the RNA-containing viruses may be regarded as a present-day version of such a primitive life form.

In this article, an attempt has been made to carry out a systematic analysis of various types of relations that may exist between the two nucleic acids. An attempt has been made to systematize the analysis and to cover most of the plausible types of interaction between these molecules. In addition, comments have been made about the possible ways in which single-stranded template mechanisms can be generalized, and a question has been raised concerning the evolutionary possibilities inherent in the existence of two closely related types of nucleic acid molecules.

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ELECTRON MICROSCOPY OF POLYNUCLEOTIDES*

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The electron microscope has provided us for many years with instrumental resolving power in the 10 to 20 Å range, which should be quite adequate for the direct observation of a great proportion of the macromolecules that are studied by the indirect methods of physical chemistry. The principal difficulty in utilizing the instrument for this kind of work arises from technical problems of specimen preparation. Organic macromolecules, being of low electron-scattering power, must have this power (or contrast) enhanced by the addition of atoms of high atomic number. The substrate structure must be finer than the dimensions of the molecules to be observed, and relative amounts of impurities and extraneous material must be kept to a very low level. In the method[1-3] that I have developed to accomplish these ends (FIGURE 1), macromolecules are suspended in a medium, usually aqueous, containing volatile salts and buffers, which is sprayed in fine droplets onto the surface of freshly cleaved mica. Polystyrene spheres of an average diameter of 880 Å are usually mixed with the sample to aid in focusing and to provide a measurement of shadow-to-height ratio. The mica surface is very smooth, being a crystal plane, and it is very hydrophilic, which causes each drop to spread over a wide area. Washing to remove salts and buffers is unnecessary because of their volatility, and this possible source of contamination is eliminated. In the second step (FIGURE 1) the surface is shadow-cast with platinum, usually at a shadow-to-height ratio of 10:1 (the filament form shown is diagrammatic; the actual form is a straight 30-mil tungsten wire on which is wound a 0.1-mm. platinum wire). The shadowed surface is backed with SiO (0.5 mg. at 10 cm.), evaporated normal to the surface and, after removal from the vacuum chamber, it is further strengthened by flowing a collodion solution (about 0.5 per cent) over the surface and allowing it to drain and dry. In the fourth and last step the surface film is scored into squares (about 3 mm. on a side) that are floated off on water. Stripping is made possible by the fact that water penetrates readily between the platinum and the mica because of the hydrophilic nature of the latter. The floating films can be picked up over conventional grids and observed in the usual fashion with the electron microscope.

The method can be applied to the study of macromolecules in general. Among the many spherical enzyme molecules that have been clearly recorded are yeast alcohol dehydrogenase (mol. wt., 140,000), alkaline phosphatase (mol. wt., 90,000), yeast alcohol dehydrogenase (mol. wt., 70,000), and pepsin (mol. wt., 35,000). Diameters of these molecules can be measured approximately from electron micrographs, but not with sufficient accuracy to compete with other methods of obtaining molecular volume. In such cases, the electron microscope's chief contribution consists in showing shape, degree of uniformity, and presence or absence of extraneous materials. The method obviously is

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most valuable for the observation of molecules having a complex morphology that cannot be deduced from hydrodynamic data. An example in this category is the molecule of fibrinogen, which appears as a string of 3 nodules or beads held together by a very fine filament.[4]

Probably the most important macromolecules to be studied by these methods are those that are highly asymmetrical.[3] Their lengths can be obtained with good accuracy even when the diameters are too small to be measured within close limits, and statistical distributions of particle lengths are readily ob-

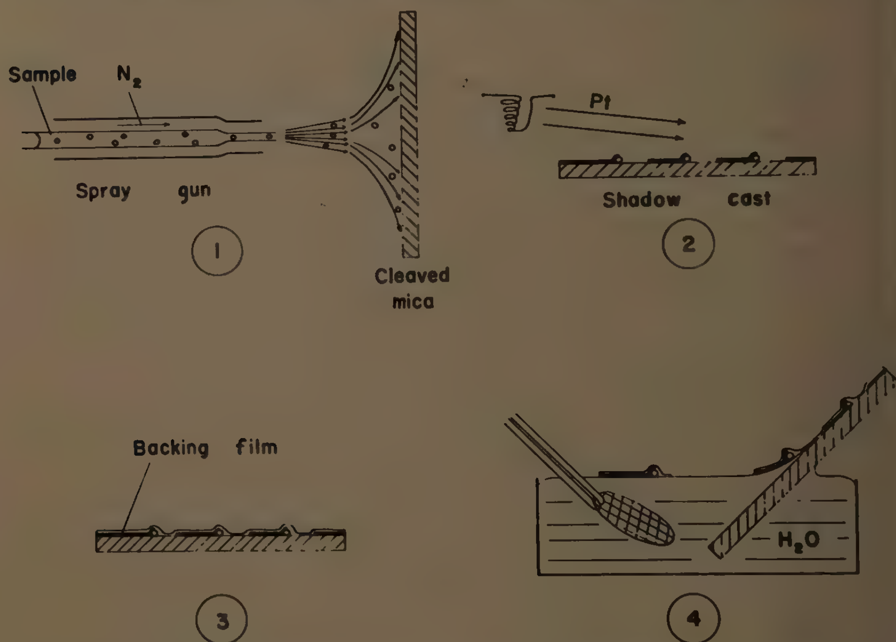

FIGURE 1. Method for preparing macromolecules for observation with the electron microscope.

tained. An example of long thin molecules are the segments of desoxyribonucleic acid (DNA)[5] shown in FIGURE 2*a*, reproduced for comparison with the synthetic polynucleotides to be discussed. One can see clearly individual molecular strands (for example, upper left) and an extremely smooth and clean background that, however, eventually shows a granular structure due to the SiO and the grain of the platinum. These molecules manifest a certain degree of stiffness, and the ends of undegraded molecules end abruptly, like those in the figure. In partially degraded preparations the molecules end in flat amorphous-appearing patches, which we believe are caused by dissociation and random coiling of the helices of the Watson-Crick double helix. Very long DNA molecules may be broken down to shorter lengths by the spray drop procedure.

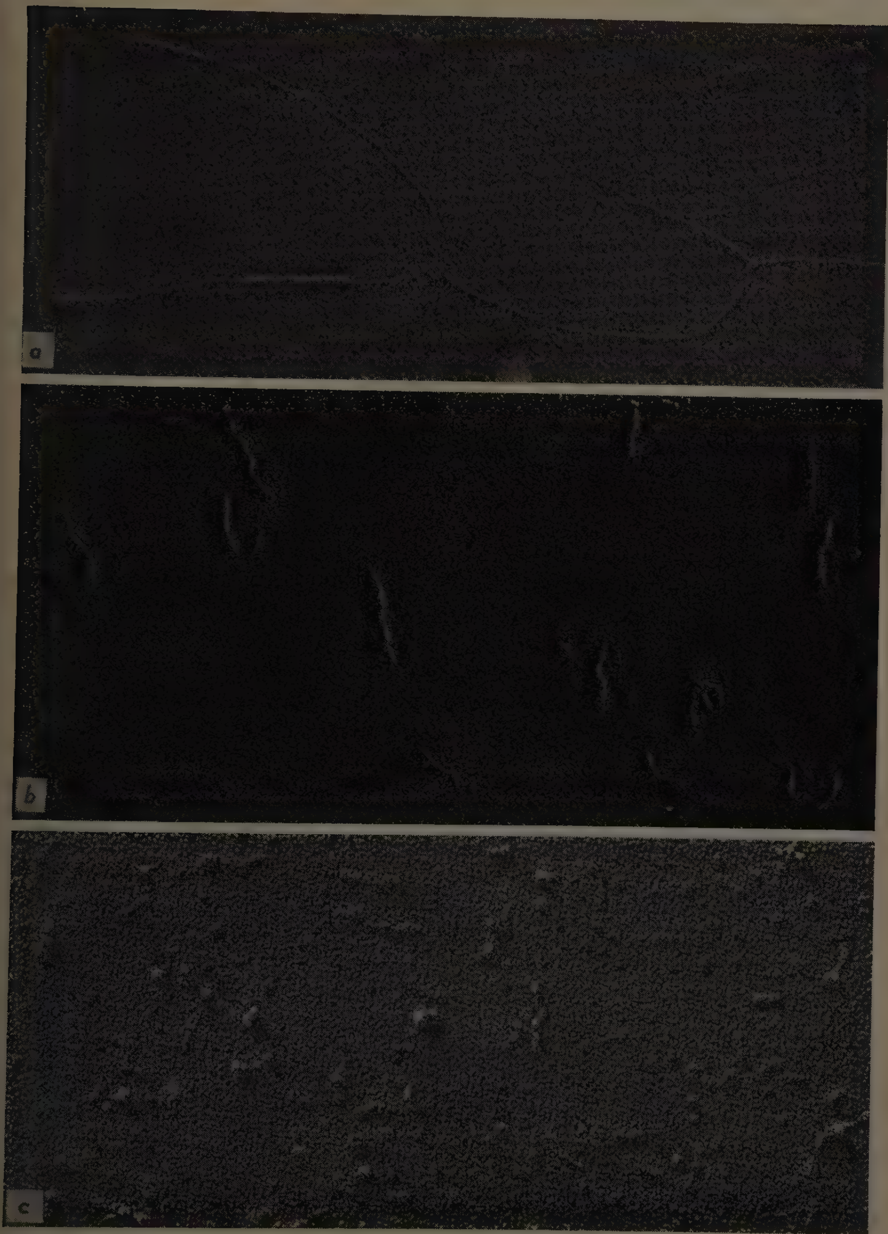

FIGURE 2. (*a*) DNA from salmon sperm. The strand at the upper left is a segment of a single molecule; at the lower left, 2 lie side by side. ×115,000. (*b*) Poly I, molecular weight about 450,000, from 1 M NH_4Cl at *p*H 4.0. ×82,000. (*c*) Poly A, molecular weight about 1,000,000, from solution in ammonium acetate-carbonate at *p*H 7.9, where the polymer exits as single-stranded random coils. ×82,000.

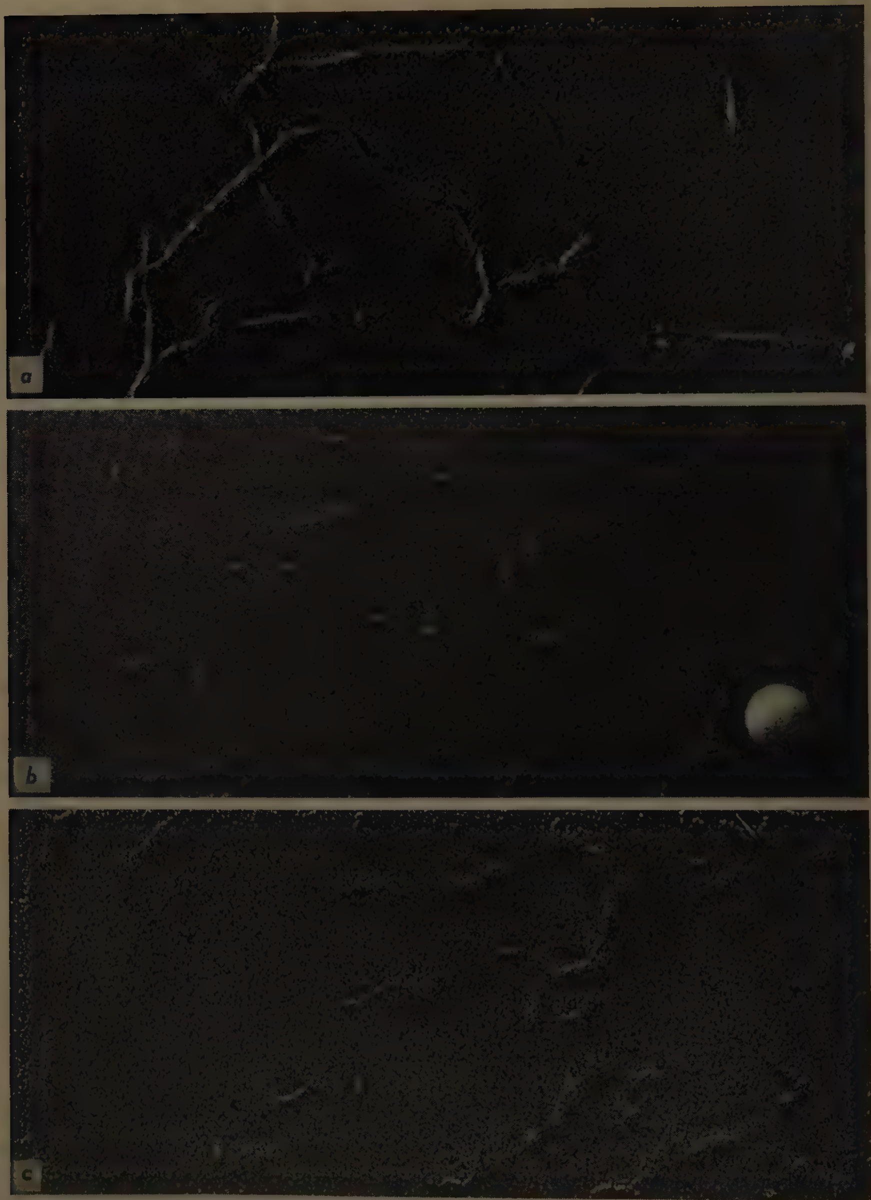

FIGURE 3. (*a*) Poly A, same as for FIGURE 2*c*, from solution at *p*H 5.1. Aggregation is caused by a trace of residual salt. ×82,000. (*b*) Poly C from solution in 0.4 M NH_4 acetate at *p*H 5.0. ×100,000. (*c*) Poly IC made by mixing amorphous poly I and amorphous poly C at a *p*H of about 6.9 in 0.1 M NH_4 acetate. ×100,000.

Observations of Polynucleotides

Polyinosynic acid (*poly I*). The first example of a polynucleotide is poly I, shown in FIGURE 2*b*. This and the other sample of poly I discussed below were made at Harvard University, Cambridge, Mass., by R. Haselkorn, who is studying them by physicochemical techniques. From electron micrographs, the weight-average length of this sample is about 1500 Å, and the number average 970 Å. It has a molecular weight of about 450,000 and the preliminary results indicate a mass per unit length corresponding to a triple strand.[6]

Another similar sample of poly I examined had a weight-average length of 500 Å and a molecular weight of about 200,000.

Polyadenylic acid (*poly A*). Electron micrographs of a number of samples of poly A have been made by J. Fresco[7] at Harvard. The first samples had a molecular weight between 100,000 and 200,000. At *p*H 8.6, one sees only a rough particulate background in which it is not possible to distinguish characteristic units. This appearance is consistent with the conclusion that at this *p*H the molecules exist as random coils of single strands. At *p*H 4.7, in

TABLE 1
ELECTRON MICROSCOPE OBSERVATIONS OF POLY I AND POLY C IN NH_4 ACETATE

	1 M *p*H 6.8–7.0	0.01 M *p*H 6.8–7.0	0.4 M	0.1 M *p*H 6.8–7.0
Poly I	Rods	Amorphous	*p*H 6.5 Amorphous	Poorly defined linear elements } Rods
Poly C		Amorphous	*p*H 5.0 Rods	Amorphous } Rods

water, one sees well-defined rods having lengths of as much as 0.5 μ. Under these conditions, Fresco has obtained X-ray lines corresponding to a double helical structure. In the presence of salt (NH_4Cl) at *p*H 4.3, one sees also well-defined rods, but there is an obvious tendency to lateral aggregation.

The micrographs in FIGURES 2*c* and 3*a* illustrate the same phenomena from another sample of poly A having a much higher molecular weight (about 1 million). In FIGURE 2*c*, made from solutions in ammonium acetate-carbonate mixture at *p*H 7.9, where the polymer exists as single-stranded random coils, we see particles of various shapes having dimensions of about what one would expect for this molecular weight. These particles are seen much more clearly than those in the lower molecular-weight sample at this *p*H. In FIGURE 3*a*, at *p*H 5.1, we see well-defined rods on a very clean background. There was a little residual salt in this preparation, which accounts for a tendency to aggregation.

Poly A at *p*H 6.5 in water showed a mixture of random coil and rod structures. Preparations from water at *p*H 7.5, where the single strands should be straightened as a result of charge effects, show mostly a very granular background and some rare straight strands.

Polycytidylic acid (*poly C*) *and* (*poly IC*). The experiments described here and summarized in TABLE 1 were suggested by A. Rich. Poly I as previously

described, exists as well-defined rods, presumably triple helices, in 1 M NH_4 acetate at *p*H about 6.9. At lower salt molarities, amorphous or very poorly defined linear elements were observed, as indicated in the table. Poly C, as shown in FIGURE 3*b*, exists as well-defined although not very long rods at *p*H 5.0 in 0.4 M salt. The widths are roughly the same as the other polynucleotides in the multiple-helix configuration. At 0.1 M salt concentration poly C appears amorphous, presumably existing as a random coil. When poly I and poly C in their amorphous states in 0.1 M salt are mixed together, however, we obtain from the mixture the well-defined rods shown in FIGURE 3*c*, which are presumably double or multiple strands of poly I and poly C combined.

Discussion

In this presentation, I have attempted to demonstrate that the electron-microscopic method is quite capable of providing visual information regarding the individual polynucleotides with which the biochemist and the physical chemist work. The advantages of the method are as follows:

(1) There is considerable satisfaction in seeing the entities, even though the observations may do little more than confirm what has been deduced from other techniques.

(2) The electron micrographs are of value in demonstrating such morphologic features as smoothness, rigidity, flexibility, and states of aggregation.

(3) The parameter most readily and accurately obtained for asymmetrical molecules is the distribution of lengths from polydisperse solutions. Widths cannot be obtained with high accuracy in the 10 to 40 Å range, but often a rough value is useful.

(4) The method is very sensitive to the presence of extraneous material. Where very clean backgrounds are obtained, one can be reasonably sure that impurities are low or, where a polymerization process is involved, that the conversion is complete.

Disadvantages of the method are as follows:

(1) Features are deduced from dried preparations that we hope to correlate with those existing in solution (drying artifacts is always possible).

(2) Large amounts of nonvolatile salts, necessary in certain biochemical solutions, cannot be tolerated. Some nonvolatile salt can be tolerated, however, perhaps to about 0.001 M. In some cases, a nonvolatile salt may become crystallized and localized in the drying pattern, leaving the molecules of interest unobscured; this has happened with NH_4Cl in some of our experiments.

(3) There is inadequate resolution of intramolecular structure, such as the multiple-helix configurations, because of the finite grain size of the shadowing metal. Some improvement may be expected in this direction, however.

(4) There is poor accuracy in the measurement of shadow lengths because of the extreme obliquity of the shadowing angle. Some improvement may be expected in the future in this area also.

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POLYRIBOADENYLIC ACID, A MOLECULAR ANALOGUE OF RIBONUCLEIC ACID AND DESOXYRIBONUCLEIC ACID*

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It seems appropriate to the theme of this monograph to discuss the present state of our knowledge of the best characterized, enzymatically synthesized polyribonucleotide, polyriboadenylic acid (poly A). This can be done profitably by considering as analogues of the two types of naturally occurring nucleic acids the two molecular configurations that poly A can assume at room temperature.

Our initial studies with poly A[1] showed that this homopolymer could exist in two forms, depending on the *p*H and ionic strength of the solution. Each form has a characteristic ultraviolet absorption spectrum shown in FIGURE 1; investigations of their molecular properties revealed the fact that each form represents the opposite end of a helix-coil transformation, schematically presented in FIGURE 2. More recently our attention has centered on the fine structure of each configuration and on a more detailed examination of the reversible transition.

Poly A at Neutral pH

In neutral saline solution (the standard solvent is 0.15 M NaCl + 0.015 M Na citrate), poly A exists as a contracted, irregularly coiled, flexible, single chain. The first indication of this came from a study of the molecular-weight dependence of the sedimentation constant and intrinsic viscosity. The dependence of these properties was clearly that predicted for randomly coiled single chains, but it was not typical for a polyelectrolyte having such a high charge density. Indeed, it appeared that a substantial proportion of the nucleotides in a single chain must be involved in some type of intrachain contact. This, taken together with the observation first reported by Warner[2] that poly A in neutral saline is hypochromic to the extent of about 35 per cent with respect to the mononucleotide, suggested to us that these contacts are hydrogen bonds between the adenine bases.

The polyelectrolyte character of poly A becomes obvious when the charge-shielding ions are lowered in concentration, as shown in FIGURE 3. Here the reduced specific viscosity shows a progressively sharpening rise for a solution of poly A whose salt concentration was lowered together with polymer concentration; in contrast to this is the small linear decrease for the same sample when maintained at constant ionic strength while its polymer concentration was reduced. Concomitant with the molecular expansion with decreasing ionic strength, we find a rise in the extinction coefficient. Evidently, exposure

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† Established Investigator of the American Heart Association, New York, N. Y.

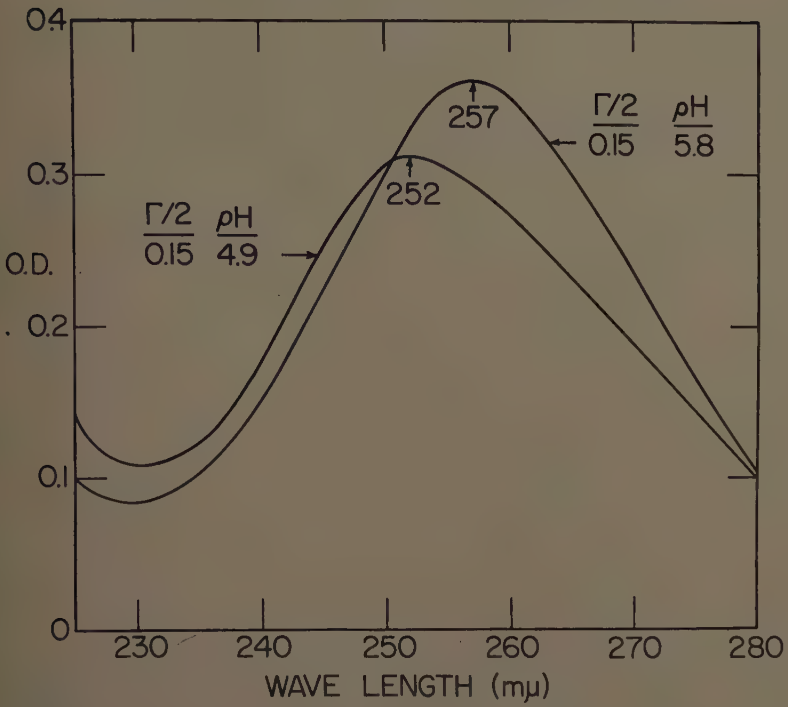

FIGURE 1. Spectra of the two configurations of poly A at the same concentration.

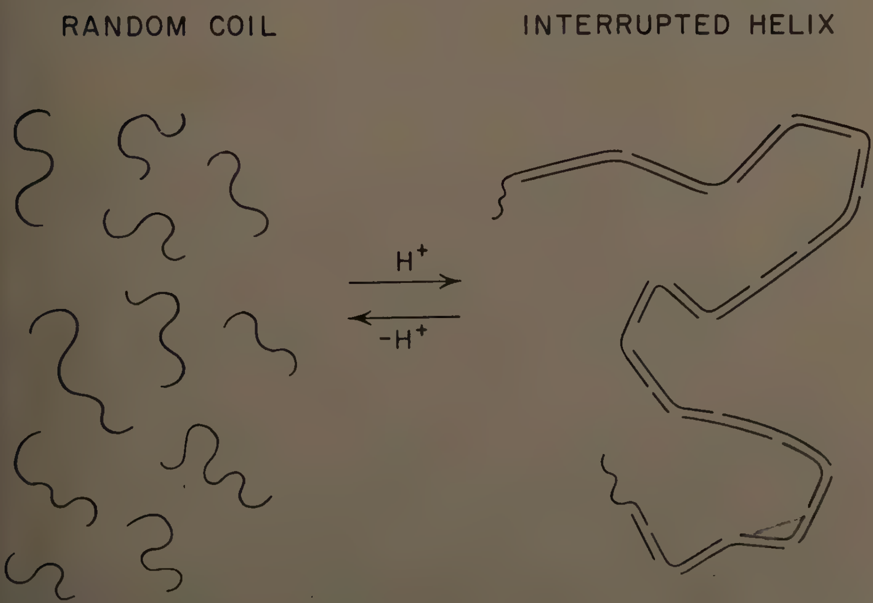

FIGURE 2. A schematic representation of the pH-induced transition of poly A.

of the charged phosphate groups increases the electrostatic energy sufficiently to overcome the binding energy of the weak intramolecular hydrogen bonds.

The energy distribution of these bonds must be quite broad, if we are to judge from the gradual loss of hypochromicity associated with increase in temperature; this is seen from the optical density–temperature profile shown

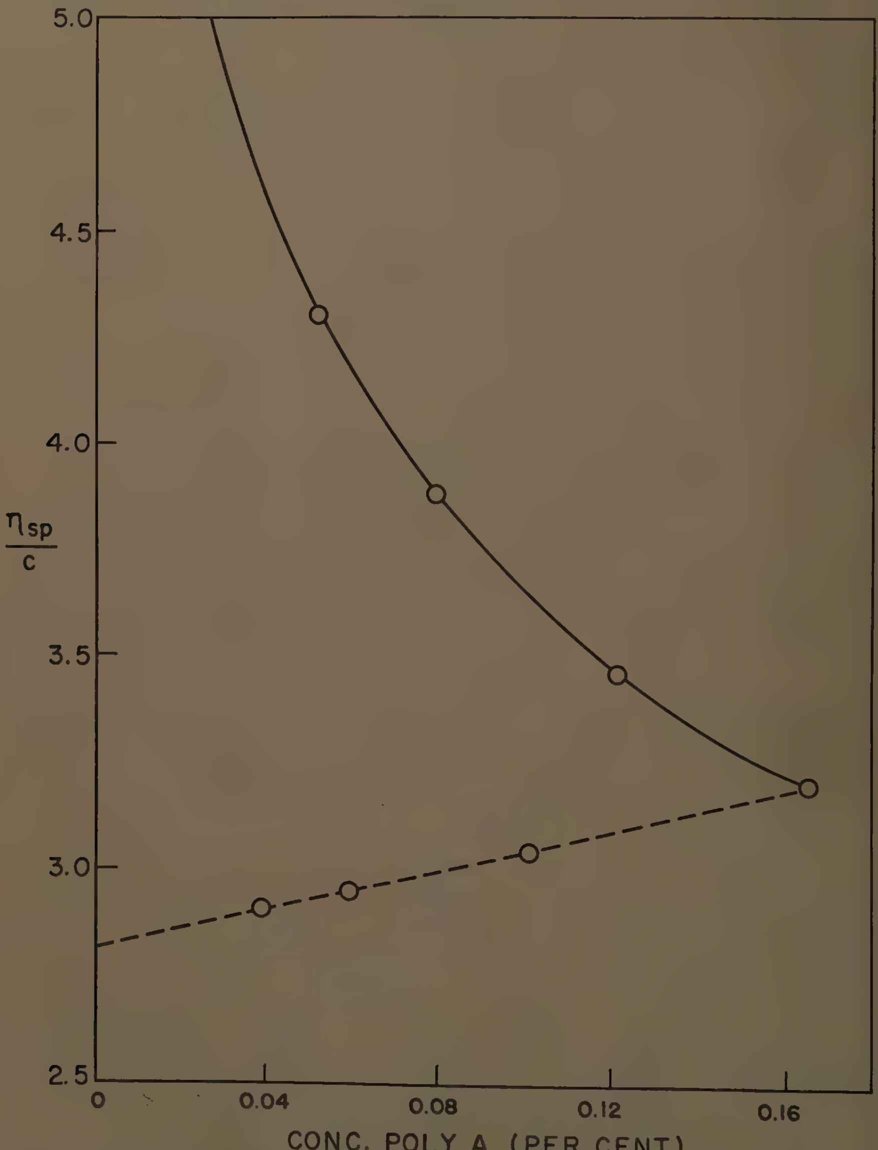

FIGURE 3. The effect of ionic strength on the reduced specific viscosity of poly A at neutral *p*H. In both cases the starting solvent is 0.15 M NaCl + 0.015 M Na citrate. The dashed line represents the solution in which ionic strength was kept constant. The points on the curved solid line were obtained on diluting the initial solution with water.

in FIGURE 4. From these data we can determine also that the hyperchromic change on going from 25° to 90° C. amounts to only a 50 per cent reversal of the hypochromicity observed in going from monomer to polymer at 25° C. It is possible to increase this reversal to a maximum of about 65 per cent with urea (*see below*). It would seem then that most of the remaining 35 per cent represents the spectral effect of forming the internucleotide bond.

Our hypothesis is, of course, that the rise in temperature brings about the dissociation of hydrogen bonds between the adenine bases. We can cite 2 observations to support this view. The first concerns the effect of urea, an agent known to disrupt hydrogen bonds of other types of interacting groups by competing with them. When urea is added to a solution of poly A to the extent of 7.2 M, about 75 per cent of the hyperchromic effect achieved on going from 25° to 90° C. is brought about at 25° C.

The second observation derives from the fact that formaldehyde reacts readily, in the *p*H range 3 to 7, with the amino group of adenine and its derivatives.[3] In the case of poly A, we find that a 20° C. rise in temperature (25° to 45°) is accompanied by a twentyfold increase in the rate of reaction with formaldehyde (which is first order), in contrast to an only sixfold increase for the nucleotide. This indicates that the rise in temperature increases the number of poly A amino groups available for reaction; we conclude that these groups were formerly involved in hydrogen bonds. However, the effect of temperature is in fact over and above that predictable from the optical density–temperature data; this becomes evident from the following considerations. We assume that 50 per cent of the bases are not hydrogen-bonded at 25° C., an estimate afforded by the optical-rotation data described below. From the optical density–temperature profile we can calculate that this amount is increased to about 65 per cent at 45° C. Therefore, with the sixfold increase in rate observed for the mononucleotide, we might expect only an eightfold increase for the polymer. The observed twentyfold increase in rate suggests, therefore, that at higher temperatures the dynamic exchange of hydrogen bonds must also be accelerated.

Doty *et al.*,[4, 5] elsewhere in this monograph, describe the basis for the interpretation of the optical rotation of polynucleotides. It is sufficient, therefore, to note that the single-chain form of poly A at 25° C. has a specific rotation intermediate (+160° to 180°) between that of the completely helical form (+300°, *see below*) and that observed at 90° for the non-hydrogen-bonded polyribouridylic acid (∼0°).[6] Further, as can be seen in FIGURE 4, the temperature-dependent variation in specific rotation is congruent with the optical-density changes.

These observations, then, are consistent with the idea of short helical regions within the single-chain form of poly A, and it is in this respect that poly A provides a simple analogue of the more complex natural polynucleotide, RNA. It follows that adenine-adenine interactions may be important in RNA.

The Nature of the Helix-Coil Transition

In acid solution, poly A chains come together to form a 2-stranded helical complex. This acid-induced transition appears to be a cooperative process. Thus we find that the titration curve of poly A is abnormally steep on the

alkaline side, about 50 per cent of the protons being picked up in less than 0.1 *p*H unit (see FIGURE 5). When the transition is complete, approximately 60 per cent of the adenine groups have been protonated, and the titration curve then follows the normal broad course for a single group in a polyelectrolyte.

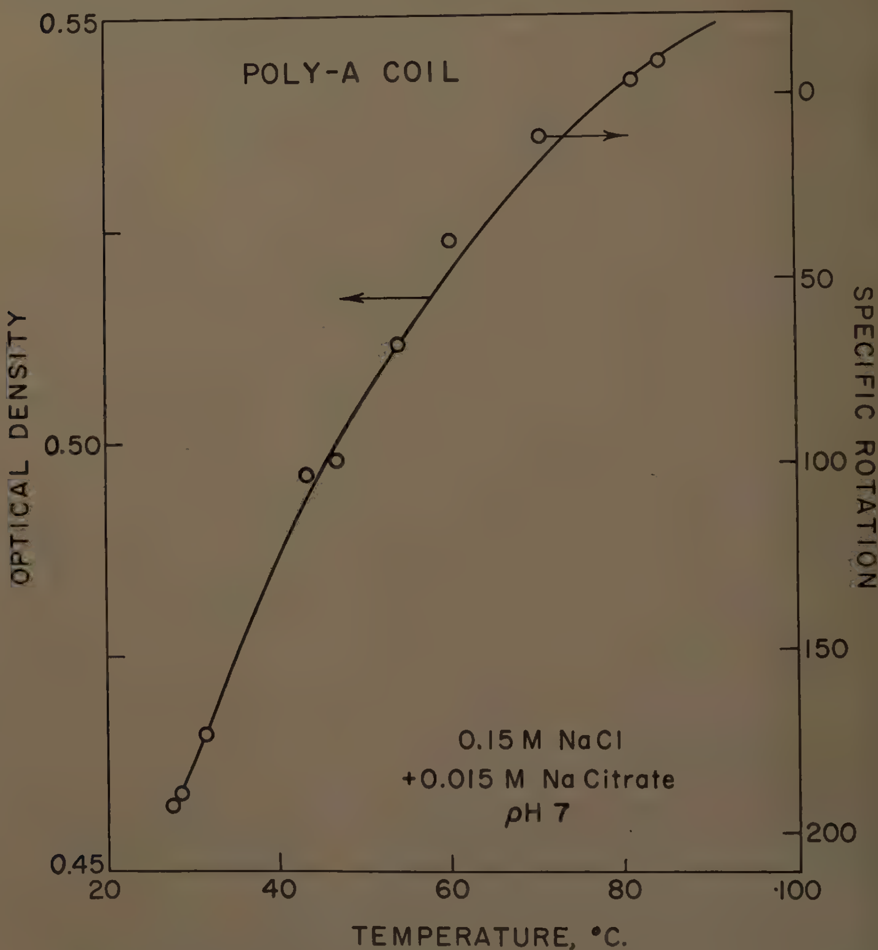

FIGURE 4. The variation in optical density (*solid line*) and specific rotation (*open circles*) in relation to temperature of neutral solutions of poly A.

Coincident with the transition is the appearance of the acid spectrum (FIGURE 1). Only over the very narrow *p*H range of transition do we observe spectra intermediate between the 2 shown in the figure.

A point of some debate has been the question whether the transition leads to the formation of a random, disordered aggregate or to an organized crystalline complex.[7] The very sharpness of the transitions suggests the formation of highly ordered and organized bonds. We present, in the following section,

other evidence from the study of physicochemical properties to support this view.

It is also worthy of note that the manner in which the transition is brought about has a marked effect on the character of the complex formed, for defects can occur that manifest themselves in the formation of aggregates whose properties mask those representative of the perfectly formed 2-stranded helices. In our experience, high ionic strength, large molecular weight, and high concentration of polymer all contribute to the aggregation phenomenon. However, with high molecular weight–single chains (about 800,000) and at a concentration of 0.15 per cent we have been able to induce essentially perfect transitions by first dialyzing away the neutral saline into water at neutral *p*H and then slowly introducing into the aqueous solution buffer at the acid *p*H

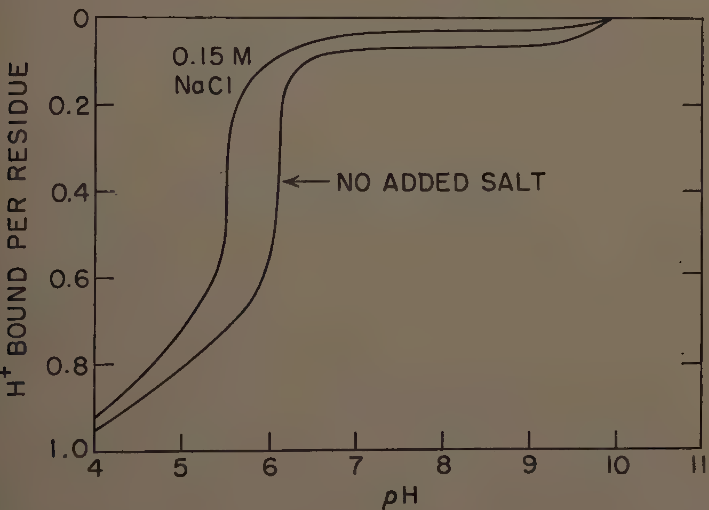

FIGURE 5. Titration curves of poly A solutions of 25° C. The curves are for solutions initially brought to *p*H 10.

until an ionic strength of 0.15 is reached again. If, instead, the *p*H is lowered while the ionic strength is high, a good deal of lateral aggregation occurs, which is evident through altered viscosity and sedimentation behavior, as well as through direct observation in the electron microscope.[8]

The transition can be reversibly induced by changing the *p*H. In these circumstances, variations in ionic strength alter the *p*H range of transition in the expected fashion (FIGURE 5). The helix → coil transition can also be brought about by using temperature as the inducing variant. In this way it has been possible to follow the course of transition spectrophotometrically, since the thermal disruption of the helical complex, even in the standard acid buffer (0.15 $\gamma/2$ acetate, *p*H 4.8 to 4.9), causes the spectrum to shift to one nearly identical to the neutral *p*H spectrum at that elevated temperature. Thus, we have traced the course of the transition by determining the hyperchromic change that occurs at 257 mμ. We have used this approach to evaluate

the effects of net charge and helix length on helix stability. As has been shown by Doty *et al.*,[4] the temperature mid-point of transition T_m, can serve as a measure of these effects.

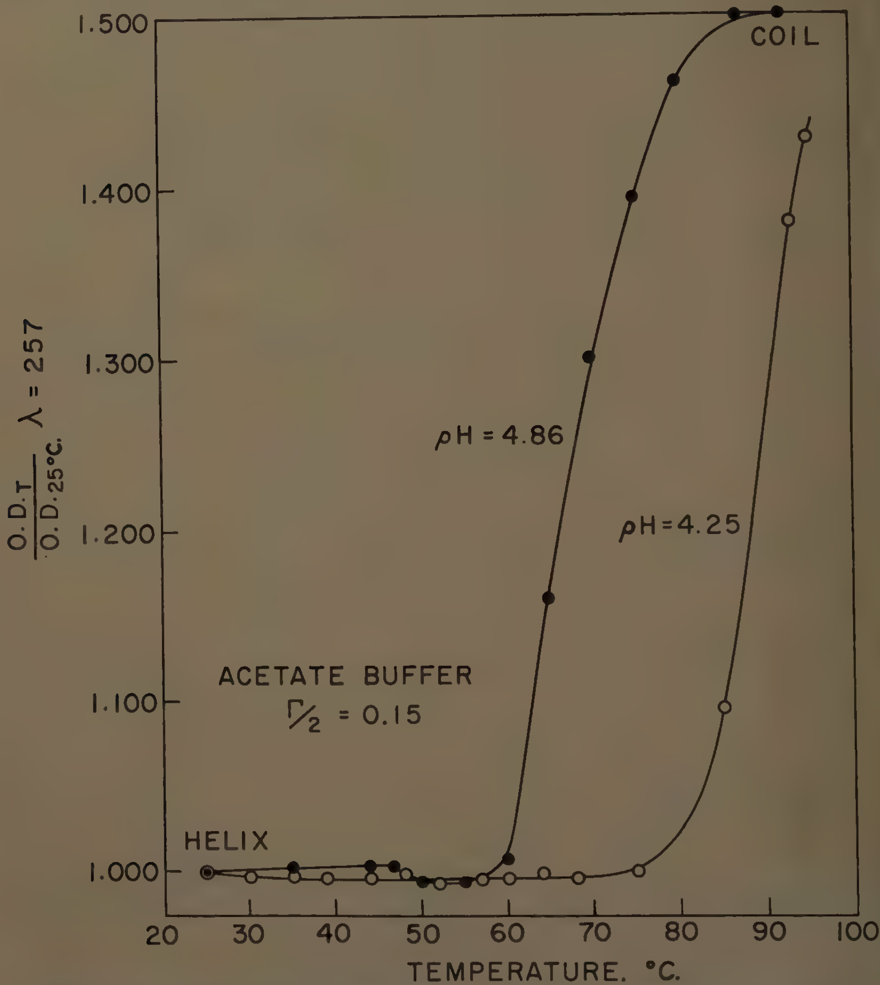

FIGURE 6. The variation in optical density as a function of temperature and acidity of solutions of poly A. Compare with FIGURE 4.

It is readily evident from FIGURES 6 and 7 that the transition is indeed sharp (occurring over a range of less than 10° C.) and, therefore, merits characterization as a type of melting process. This provides further evidence that the configurational change involves a cooperative mechanism. The effect of lowering the *p*H is to protonate the adenine groups of poly A and thereby reduce the net charge. On going from *p*H 4.86 to 4.25, this effect (FIGURE 6) is seen to be reflected by a rise in the T_m of about 20° C. Increase in the

length of helix has a similar effect, but one of lesser magnitude. This is seen in FIGURE 7, where the curves are for samples with molecular weights that increase (from left to right) in steps of about 2. It is noteworthy also that the

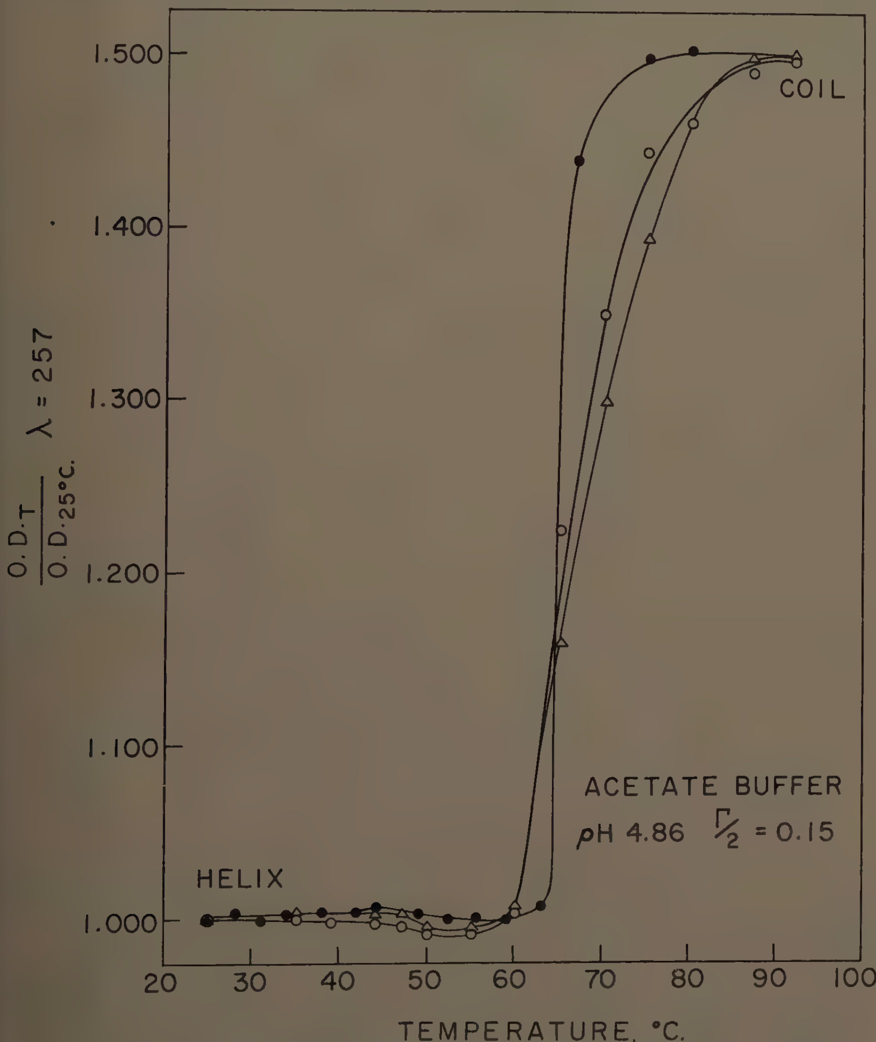

FIGURE 7. The variation in optical density as a function of the temperature of solutions of poly A complexes that vary in size. *Filled circles* are for the smallest and *triangles* for the largest complexes.

smallest helical complex displayed the sharpest transition, an observation consistent with the fact that this sample showed the least polydisperse sedimentation-boundary profile. Finally we point out that, barring hydrolytic damage, the transition induced by temperature essentially is reversed on cooling.

Poly A at Acid pH

Having referred above to the acid form of poly A as a two-chained interrupted helical complex, we shall examine below the evidence on which this is based, and the analogy of this form with DNA.

Our first observation was that acid poly A displayed a different spectrum in acid solution.[1] This spectrum, with its maximum at 252 mμ, is about 10 per cent more hypochromic with respect to the mononucleotide than that of the neutral *p*H form. This increased hypochromicity suggested that the acid form of poly A is a more extensively hydrogen-bonded structure, a view strengthened when it was observed that, in acid solution, poly A shows little increase in viscosity on lowering the ionic strength. Such acid solutions also exhibit marked negative birefringence of flow, in contrast to those at neutral *p*H, indicating that the bases are oriented essentially perpendicular to the chain axis. Clarification of the structure also developed from the finding that the acid-induced transition is accompanied by an increase in molecular weight (more properly, particle weight) in a manner proportional to the concentration of polymer. Taking advantage of this, we prepared, from a single sample of polymer, a series of complexes spanning a fortyfold range in particle weight. The study of their sedimentation and viscosity properties revealed that these complexes are homologous polymers very similar to sonic fragments* of DNA.[9] The differences in the molecular weights of the poly A complexes could be the result only of linear growth occurring by chain overlap (see FIGURE 2). The relative rigidity and highly extended character of the complexes indicate that the chain overlap is not restricted to the ends, but rather encompasses nearly the entire length of the chains, leaving very short gaps. The process of linear growth occurs prominently under appropriate conditions during the transition. Once formed, the complexes are relatively stable in the standard acid solvent for periods at least sufficiently long to permit their physical properties to be determined. However, for high-molecular weight polymer samples, the growth process has been observed to proceed slowly over many months, so that sometimes gels eventually form.

The argument just presented is not sufficient to allow us to conclude that the complex is two-stranded over most of its length, rather than three- or four-stranded. At first, a two-stranded model was assumed merely because of simplicity and its resemblance to DNA; it remained for crystallographic evidence to prove this.[10, 11, 13, 14]

The orientation of the adenine groups, revealed by the negative birefringence and the highly ordered arrangement of secondary bonds (indicated by the cooperative nature of the transition), can readily be seen to be the structural consequences of interchain interaction, if it is due to the periodic arrangement of hydrogen bonds between adenine groups of opposite chains. The reaction of acid poly A with formaldehyde, used to explore this possibility, is conveniently followed spectrophotometrically, since formylation of adenine results in a spectral shift to higher wavelengths, and a small increase in extinction. In FIGURE 8 are shown the spectrum for an acid solution of poly A and the altered

* Sonic fragments of DNA remain double-stranded helical chains whose lengths are shorter than those of the parent molecules because of sonically induced double-chain scissions.

spectra of solutions of equal concentration at different *p*Hs after incubation with formaldehyde for 2 hours and 20 hours, respectively. As expected (*see above*), the solution at *p*H 7.35 (coil form) reacts quickly and completely in 2 hours at 44° C. At *p*H 4.1 there is very little reaction after 2 hours, and hardly any in the next 18 hours. As the *p*H is raised, however, even in the region below the transition, the reaction rate is accelerated. The reaction of the

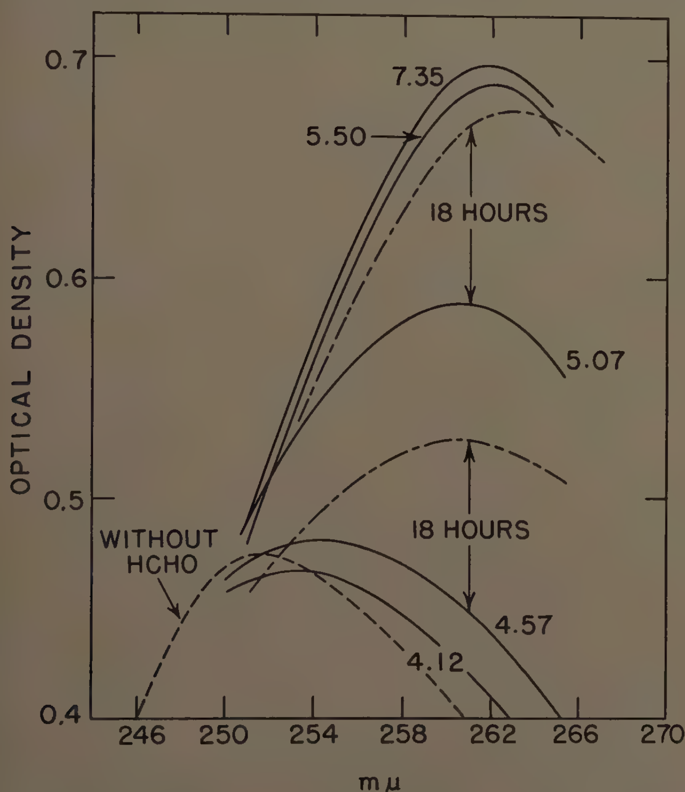

FIGURE 8. The *p*H dependence of the reaction of poly A with formaldehyde at 44° C. The spectra are for solutions at 0.1 ionic strength, all at the same concentration of polymer. *Solid lines* indicate spectra of solutions after 2 hours' incubation; *dashed lines*, 18 hours later.

monomer with formaldehyde does not show this rate dependence on *p*H, and it is not accompanied by the large hyperchromic change observed with poly A. Hence, the reaction of poly A with formaldehyde (at any *p*H) is probably accompanied by the disruption of hydrogen bonds involving the reactive amino group. At a *p*H near 4, where the electrostatic repulsion of the negative groups is almost eliminated, nearly all the adenine residues must be involved in hydrogen bonds. Indeed, the complex is so stable that formylation of the few nonhydrogen-bonded adenine residues, which are presumably at chain ends or at gaps, does not strain the structure sufficiently to open the interacting chains. As the *p*H rises (below the *p*H of transition), the number

of initially available adenine groups increases, and the strain arising from these formylated groups is sufficient ultimately to break all the hydrogen bonds.

Evidence for the helical nature of the complex has been derived from two types of solution studies. Dilute acid solutions of poly A afford remarkably sharp X-ray diffraction patterns[10] (in contrast to complete absence of diffraction by solutions at neutral *p*H) that resemble very closely the fiber diffraction pattern.[11] This pattern, with its paucity of meridional spacings, bears the unmistakable mark of a helical structure. The very high specific rotation of acid solutions of poly A ($[\alpha]_D$ is about $+300°$) provides the second type of evidence. In view of the detailed consideration of optical rotation in the paper by Doty *et al.*,[4] we need mention only that this specific rotation is about twice as great as that observed for solutions of poly A at neutral *p*H, and that it decreases in a manner coincident with other properties that reflect the helix-coil transition.

These observations on the solution properties of the acid form of poly A are consistent with the two-chain helical model deduced from crystallographic evidence by Crick *et al.*[14], Watson,[13] and Rich.[11] In this model the chains are held together by the cohesive force of hydrogen bonds between adenine residues of opposite chains, the base pairs being in the interior and the phosphate groups on the periphery of the helix. It appears from our observations that the protonation of the adenine residues, which we believe occurs at $N - 1$[10], not only reduces the net charge, but also makes possible additonal stabilizing interactions. These presumably could take the form of salt linkages between the positive site on the adenine and the negative one on a phosphate group of the adjacent chain. If such interactions were of little importance we might expect the completely helical form to exist at high ionic strength in neutral solution, where the negative charges would be shielded by the ionic atmosphere. We have not found this to be the case; in this connection, it should be emphasized that the helical regions that we propose for the random-coil form of poly A cannot involve the same type of adenine-adenine bond that occurs in the completely helical acid form of poly A.

The analogy between the acid form of poly A and DNA is by now obvious. In particular, it may be recalled that the poly A helix is much like the interrupted helical model once proposed for DNA by Dekker and Schachman.[12]

Acknowledgments

This investigation is the result of a collaboration with Paul Doty. The expert technical assistance of Richard Blake is also acknowledged.

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OBSERVATIONS UPON COPOLYMERS OF ADENYLIC AND URIDYLIC ACIDS*

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In the following contribution it is desired to dwell briefly upon some preliminary results bearing upon the stability of multistranded helical polynucleotide molecules. From the work of several laboratories it appears to be well established that polyriboadenylic acid (poly A) and polyribouridylic acid (poly U) can interact to form, depending upon conditions, either a doubly (AU) or a triply (AU_2) stranded helical complex.[1-3] The former appears to have many structural points of analogy to native desoxyribonucleic acid (DNA), the adenine-uracil hydrogen bonded pairs being very similar to the adenine-thymine pairs occurring in DNA.

Inasmuch as there is no evidence for the occurrence of any uracil-uracil hydrogen bonding in poly U, at least in solution, it is highly probable that the introduction of uridylic residues into the poly A chain would result in interruptions in any multistranded complex subsequently formed by the interaction of the copolymer with poly U. If the doubly stranded helix formed under these circumstances can be compared to a twisted ladder in which the rungs represent hydrogen bonds, the complex formed by the copolymer would resemble such a ladder with rungs missing at each point at which a uridylic residue occurred in the copolymer strand of the helix.

It was desired to ascertain the maximum degree of substitution of poly A with uridylic residues consistent with the formation of stable multistranded complexes with poly U. Copolymers of adenylic and uridylic acids were prepared in varying base ratios by the action of the nucleotide-polymerizing enzyme isolated from *M. lysodeikticus* upon mixtures of the corresponding nucleotide diphosphates.[4]

None of the copolymers thus prepared showed any important sudden drop in molecular weight at *p*H 10.5 from the value at neutral *p*H. As all uridylic residues (*p*K $\sim$ 9.5) should have lost a hydrogen ion at the former *p*H, it appears unlikely that intermolecular hydrogen bonding is important for these copolymers.

FIGURE 1 shows the change in relative absorbency, at 259 mμ, from the expected mean for the nonassociated components for mixtures of adenylic-uridylic copolymers (poly AU) with poly U. A pronounced interaction occurs at neutral *p*H (ionic strength 0.1, 25° C.) for copolymers containing mole fractions of uridylic residues of 0.18 and 0.38. The variation in absorbency is linear for mole fractions of poly U <0.50. A downward bulge in the curve at higher mole fractions of poly U indicates that the AU_2 complex as well as the AU is formed to some extent. FIGURE 1 (*insert*) compares the temperature variation of absorbency for 1:1 mixtures of poly U with poly A, the poly AU

* The opinions or assertions contained in this article are those of the author and are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large.

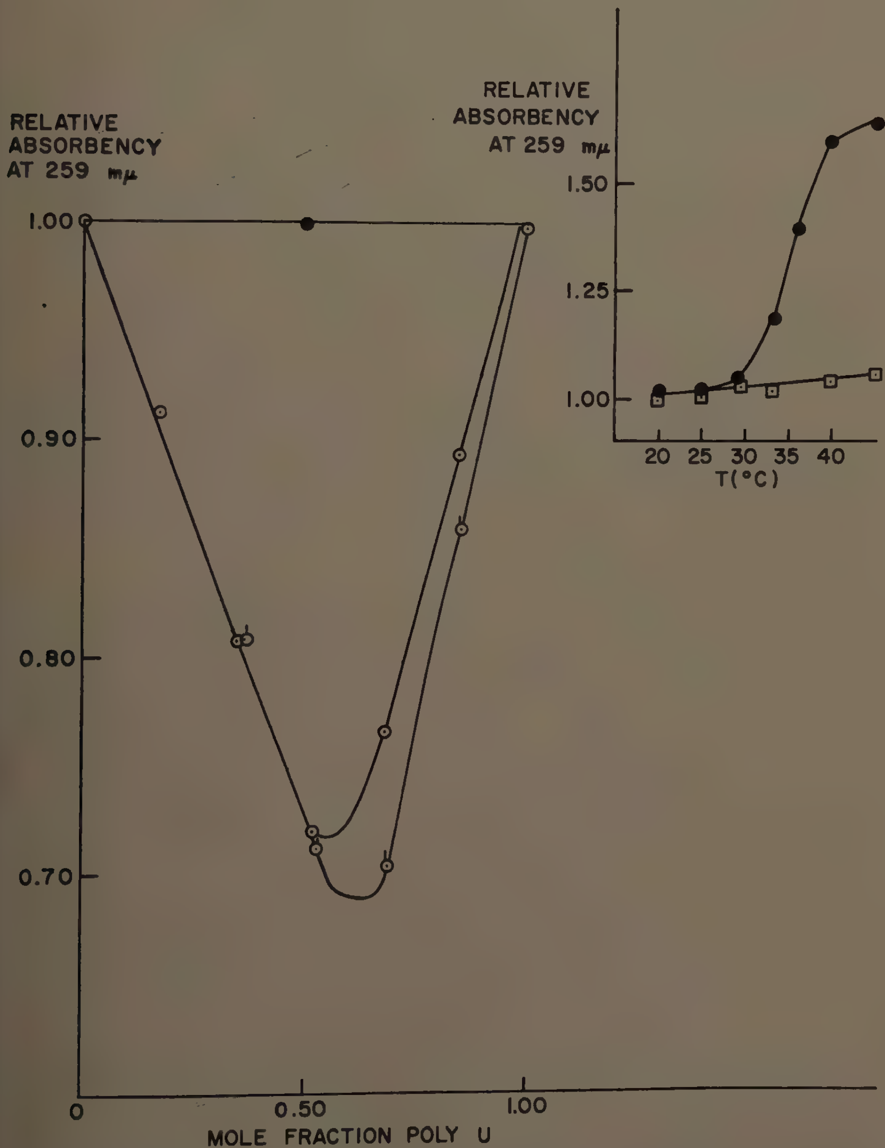

FIGURE 1. Relative absorbency at 259 mμ as a function of composition for mixtures of poly U VI ($S_o^{20°} = 8.5 \times 10^{-13}$) with several AU copolymers in 0.1 M KCl and 0.01 M NaOAc, *p*H 6.5 at 25° C. Symbols: ⦶, poly AU V, 18 per cent uridylic residues, $S_o^{20°} = 8.3 \times 10^{-13}$; ⊙, poly AU VII, 38 per cent uridylic residues, $S_o^{20°} = 4.6 \times 10^{-13}$; ●, poly AU VI, 50 per cent uridylic residues, $S_o^{20°} = 2.7 \times 10^{-13}$. The insert shows relative absorbency as a function of temperature for equimolar mixtures of poly U VI with poly A and with a copolymer. The solvent is the same as above. Symbols: ⊡, poly A XX; ●, poly AU VII.

containing 38 per cent uridylic residues. Dilution of the poly A chain to this extent has served to lower the "melting point" of the complex from about 55° C. to 35° to 38° C. at this ionic strength.

Substitution of the poly A chain with about 50 per cent uridylic residues was sufficient to block completely any interaction with poly U, as FIGURE 1 shows.

The preceding results are still in the process of evaluation, and it is hoped to develop their consequences more fully in a subsequent report. It seems very likely that the complexes formed by the copolymers with poly U are similar in nature to those formed by poly A itself, although there is yet no direct proof. If this is true, then the inference is plain that the AU and AU_2 complexes can withstand remarkable degrees of interruption. It seems reasonable to conclude that the requirements of multistranded helical structures of this type may be less rigid than is commonly supposed. This question is of particular interest in connection with the possible occurrences of faults in the structure of native DNA and RNA. It must be pointed out, however, that a random arrangement of adenylic and uridylic residues has not yet been proved for AU copolymers prepared with the above enzyme.

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Part V. Enzymes of Polydesoxyribonucleotide Metabolism

ENZYMATIC SYNTHESIS OF DESOXYRIBONUCLEIC ACID*

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Our studies during the past 3 years on the synthesis of desoxyribonucleic acid (DNA) by an enzyme that we have partially purified from *Escherichia coli* have revealed the following basic features of this reaction.

(1) The desoxynucleoside triphosphates† of the 4 predominant bases found in DNA—adenine, guanine, thymine, and cytosine—must be present for appreciable synthesis to occur.

(2) Polymerized DNA and Mg^{++} are essential for the reaction.

(3) Inorganic pyrophosphate is released in amounts equal to the amount of nucleotide incorporated into DNA.[1-3]

On the basis of these findings, the over-all equation for the synthesis of DNA by this enzyme may be formulated in the following way:

$$n\begin{bmatrix}\text{dTPPP}\\ \text{dGPPP}\\ \text{dAPPP}\\ \text{dCPPP}\end{bmatrix} + \text{DNA} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \text{DNA} - \begin{bmatrix}\text{dTP}\\ \text{dGP}\\ \text{dAP}\\ \text{dCP}\end{bmatrix}_n + 4(n)\text{PP}$$

In this report I shall describe in some detail experiments in which we have attempted a characterization of the enzymatically synthesized DNA with regard to its physical and chemical properties. In addition, evidence will be presented for the enzymatic synthesis, under rather specialized conditions, of a DNA-like copolymer of desoxyadenylate and thymidylate.

This enzyme, which in the presence of a DNA primer catalyzes the polymerization of the 4 desoxynucleoside triphosphates, has been purified about two to four thousandfold relative to the crude extract of *E. coli* that serves as its source material. With such preparations net synthesis of DNA can be demonstrated readily by a variety of procedures (TABLE 1). In the first experiment shown in the table, an increase in DNA of somewhat more than twofold was observed as measured by isotope incorporation, ultraviolet spectrophotometry, or desoxypentose estimation. In the other 3 experiments there were increases in DNA of from ten- to twentyfold, so that 90 to 95 per cent of the isolated DNA had its origin in the desoxynucleoside triphosphates supplied in the reaction. Since the enzymatically synthesized DNA shows the very high viscosity characteristic of DNA isolated from natural sources,[3, 4] one can measure the net synthesis of DNA in yet another way—that is, by viscometry. Thus, one simply incubates the various components of the reaction in a conventional Ostwald viscometer placed in a thermostated bath, and follows the

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† The abbreviations used in this report are as follows: dATP or dAPPP, desoxyadenosine triphosphate; dCTP or dCPPP, desoxycytidine triphosphate; dGTP or dGPPP, desoxyguanosine triphosphate; and dTTP or dTPPP, thymidine triphosphate.

increase in viscosity as a function of time. As shown in FIGURE 1, the viscosity of the reaction mixture continued to increase for approximately 3 hours, then stopped abruptly, probably as a result of the exhaustion of the substrates; it then underwent a gradual decline. This slow decrease in viscosity is most probably attributable to the presence of a small amount of desoxyribonuclease activity that is present even in our most highly purified enzyme preparations.

The availability of enzymatically synthesized DNA having 90 to 95 per cent of its origin in the desoxynucleotide substrates has permitted a study of its

TABLE 1
NET SYNTHESIS OF DNA

Experiment*	Estimation	Control (no enzyme) μmoles	Complete μmoles	Δ μmoles
1	P^{32} incorporation	0.00	0.28	0.28
	Optical density	0.19	0.46	0.27
	Desoxypentose	0.19	0.40	0.21
2	Optical density	0.06	0.63	0.57
3	Optical density	0.05	0.58	0.53
4	Optical density	0.05	0.64	0.59
5	Optical density	0.04	0.87	0.85

* In Experiment 1, the incubation mixture (3.0 ml.) contained 0.15 μmole of $dAP^{32}PP$ (1.3×10^6 cpm/μmole), 0.3 μmole of dGTP, 0.15 μmole of dCTP, 0.15 μmole of dTTP, 200 μmoles of potassium phosphate buffer (pH 7.4), 20 μmoles of $MgCl_2$, 0.1 mg. of calf thymus DNA, and 12 μg. of Enzyme Fraction VII.[2] The mixture was incubated at 37° C. for 180 min. DNA was precipitated, washed, taken up in 1.2 ml. of 0.5 N perchloric acid, and heated for 15 min. in a boiling-water bath. Optical density measurements were made at 260 mμ and converted to nucleotide equivalents, using a molar extinction coefficient of 8960 (derived from the calculated values for an acid hydrolyzate of calf thymus DNA). In the P^{32} estimation of DNA synthesis, incorporation of desoxyadenylate was multiplied by a factor based on its percentage composition in calf thymus DNA. The radioactivity actually observed for the controls did not exceed the background count. In Experiments 2, 3, 4, and 5, the reaction mixture (1.0 ml.) contained 0.32 μmole of each of the 4 triphosphates, 30 μg. of calf thymus DNA, 60 μmoles of potassium phosphate buffer (pH 7.4), 6 μmoles of $MgCl_2$, and 8 μg. of Enzyme Fraction VII. The mixture was incubated for 250 min at 37° C., and 2 M NaCl was then added to give a final concentration of 0.2 M; the mixture was heated for 5 min. at 70° C. Unreacted triphosphates were removed by exhaustive dialysis against 0.2 M NaCl. The product contained no acid-soluble material. Optical density at 260 mμ was determined and converted to nucleotide equivalents using a molar extinction for DNA of 6900.

physical dimensions.[4] In general, such studies have shown that the enzymatically produced material has essentially the same properties as DNA carefully isolated from natural sources. These results are summarized in TABLE 2. The average sedimentation coefficients observed for a large number of runs ranged from 20 to 25 Svedberg units. Measurements of reduced viscosity have yielded values of 15 to 45 dl./gm., which are comparable to those observed for calf thymus DNA (40 to 50 dl./gm.). On the basis of these parameters, we have calculated average molecular weights of from 4 to 6 million for a number of synthetic products. The viscosity and sedimentation behavior of enzymatically synthesized DNA would suggest that it is organized as relatively stiff macromolecules, with effective volumes that are greater than would be

expected from single polynucleotide chains with freedom of rotation at each link in the backbone. Supporting this view are the observations made when the DNA product was heated at 100° C. for 15 min. Under these conditions, the rigid structure of DNA is known to collapse.[5] Like calf thymus DNA, the viscosity of the enzymatic product decreased to less than 1 (grams per 100 ml.)$^{-1}$ and the sedimentation rate decreased only slightly to 14 S. Further-

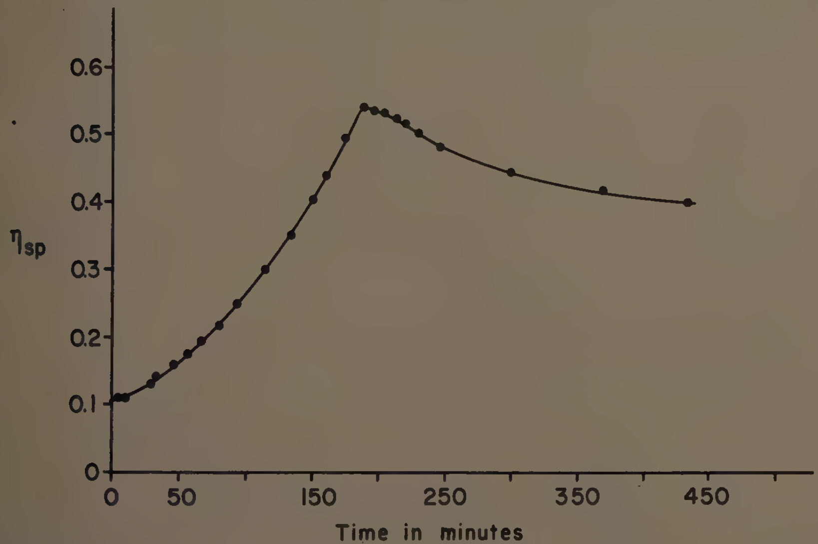

FIGURE 1. Measurement of DNA synthesis by viscometry. The incubation mixture was the same as that used in experiments 2 to 5, TABLE 1. Incubation was at 37° C.

TABLE 2
PHYSICAL PROPERTIES OF ENZYMATICALLY SYNTHESIZED DNA

	Primer	Product	Heated at 100° C., 15 min.	
			Primer	Product
Sedimentation coefficient	25	20–25	20	14
Intrinsic viscosity, dl./gm.	40–50	15–45	<1	<1
Molecular weight	8×10^6	$4–6 \times 10^6$		

more, before heating, the product showed the typical hypochromicity characteristic of a highly ordered DNA molecule.[6] Upon digestion with crystalline pancreatic desoxyribonuclease, an increase in ultraviolet absorption was produced at the same rate and to the same extent (30 per cent above the starting value) as that observed for calf thymus DNA (FIGURE 2).

With respect to the chemical composition of the enzymatically synthesized DNA, it has already been demonstrated that this DNA consists of desoxynucleotides linked by typical 3′-5′ phosphodiester bonds, and all 4 of the desoxynucleotides that occur naturally in DNA are present.[3] The Watson-

Crick[7, 8] model for DNA, however, would predict that certain base substitutions could be made provided the proper hydrogen-bonding relationships are maintained. Thus, one would expect that thymine could be replaced by uracil or bromouracil, since both of these analogues maintain the keto grouping in the 6 position and the H in the 1 position necessary for hydrogen bonding with the corresponding 6 amino and 1-N groups of adenine (FIGURE 3). Similarly, methylcytosine or bromocytosine might substitute for cytosine, and hypoxanthine may replace guanine. In the experiment summarized in TABLE 3

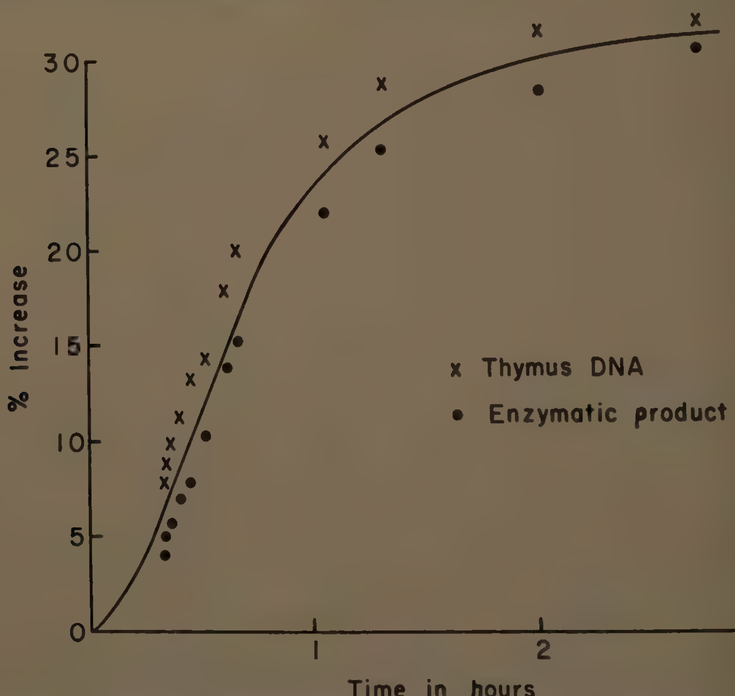

FIGURE 2. Increase in ultraviolet absorption of DNA upon digestion with pancreatic desoxyribonuclease.

the desoxynucleoside triphosphates of the various purine and pyrimidine analogues were prepared chemically and tested for their ability to replace dATP, dCTP, dGTP, and dTTP as substrates in the enzymatic synthesis of DNA. As indicated, the triphosphates of 5-bromodesoxyuridine and desoxyuridine replaced only dTTP. Similarly, 5-methyldesoxycytidylate and 5-bromodesoxycytidylate were incorporated specifically in place of dCTP. Desoxyinosinate replaced only desoxyguanylate, although at an appreciably reduced rate. These results, then, are clearly in accord with the specific base-pairing requirements of the Watson-Crick theory.

Two further questions can be posed with regard to the chemical composition of the enzymatically synthesized DNA: (1) Does this DNA show the equivalence of purines to pyrimidines observed in all naturally occurring DNAs?[9]

(2) Does the base composition of the DNA primer influence the composition of the synthesized product? To answer these questions a number of products were prepared, differing only in the source of DNA used as primer in the synthesis. The base compositions of the various primers and products were determined by the method of Wyatt and Cohen;[10] the results of these analyses

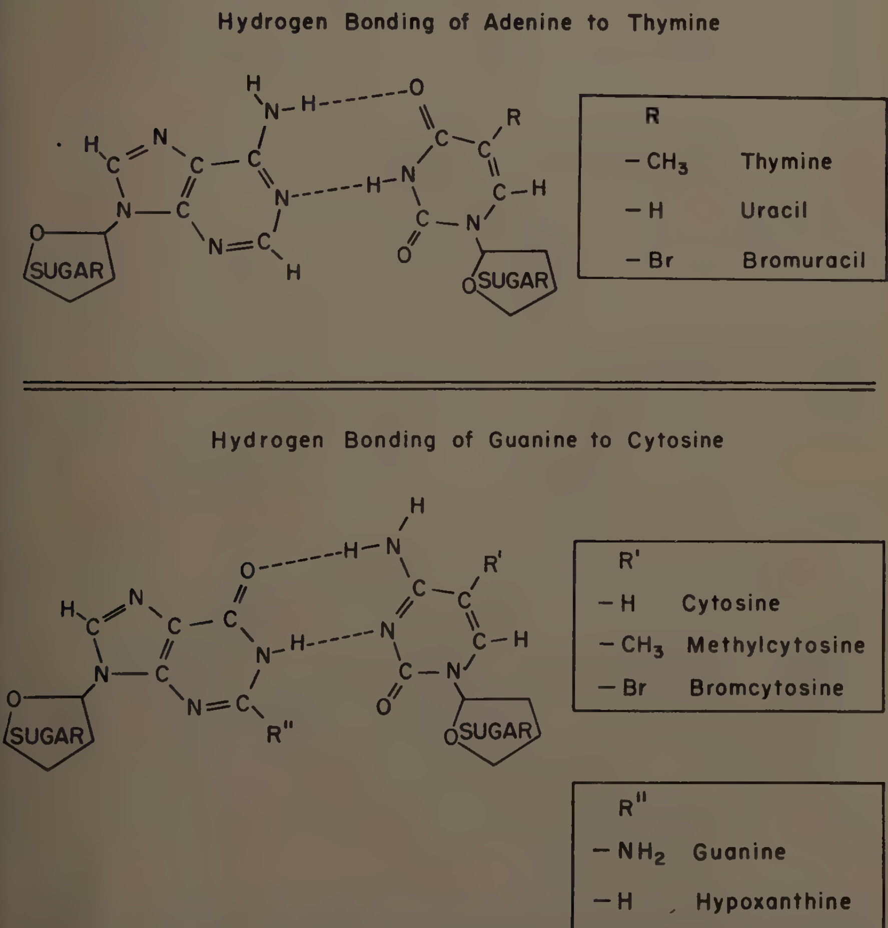

FIGURE 3. Hydrogen bonding of "natural" and "unnatural" purine and pyrimidine bases.

are summarized in TABLE 4. In each enzymatically synthesized DNA sample a close correspondence was observed between the content of adenine and thymine on the one hand, and guanine and cytosine on the other, so that the total amount of purine was in each case the same as the total amount of pyrimidine (that is, A = T; G = C; A + G = T + C). Furthermore, the ratio A+T/G+C in the enzymatic product was always close to that of the primer used in its synthesis. The primer values ranged from 0.49 for *M. phlei* to greater than 40 for the enzymatically synthesized copolymer of desoxyadenylate

and thymidylate. It is noteworthy that the net increase in DNA was in each case at least 10 times the primer added except in the *M. phlei*, where it was only fourfold. Thus, in all but the *M. phlei* samples, more than 90 per cent of the bases was derived from the nucleotide substrates for the reaction. It may be noted in TABLE 4 that the ratio of A+T/G+C was somewhat higher than that for the primer in some products; lower ratios have never been observed. It seems most probable that these divergencies between primer and product values are the result of contamination of the products with the desoxyadenylate-thymidylate (A-T) copolymer, which is produced after lag periods of from

TABLE 3

REPLACEMENT OF NATURAL BASES BY ANALOGUES IN ENZYMATIC SYNTHESIS OF DNA

Experiment	Control value* (mμmoles)	Base analogue used	Natural base omitted			
			Thymine	Adenine	Guanine	Cytosine
			(percentages of control)†			
1	0.50	Uracil	54	4	6	
1a	0.88	Uracil				3
2	0.43	5-Bromouracil	97	2	4	
2a	0.42	5-Bromouracil				4
3	0.51	5-Bromocytosine		4	4	118
3a	0.40	5-Bromocytosine	4			
4	0.58	5-Methylcytosine		2	3	185
4a	0.52	5-Methylcytosine	2			
5	0.37	Hypoxanthine		3	25	5
5a	0.27	Hypoxanthine	4			

* Control values are millimicromoles of radioactive desoxynucleotide incorporated into DNA in the absence of analogue. Incubation mixtures contained, in 0.3 ml., 5 mμmoles each of dTTP, dATP, dCTP, and dGTP; 2 μmoles of $MgCl_2$; 20 μmoles of potassium phosphate (*p*H 7.4); 10 μg. of calf thymus DNA; and 1 μg. of Enzyme Fraction VII.[2] Labeled substrates were: $dCP^{32}PP$ in Experiments 1, 2, and 5a; $TP^{32}PP$ in Experiments 1a, 3, 4, and 5; and $dGP^{32}PP$ in Experiments 2a, 3a, 4a. After incubation for 30 min. at 37° C., incorporation of isotope into DNA was measured as reported for assay of polymerase.[2]

† The percentage value represents the fraction of the labeled substrate incorporated when the analogue (5 mμmoles) was used instead of a base. All bases, natural or analogue, were supplied as the desoxynucleoside triphosphate. Values of 5 per cent or less are near the limit of detectability and are of questionable significance.

3 to 6 hours. The synthesis and properties of this polymer will be discussed in more detail below.

The results shown in TABLE 4 were obtained with equimolar concentrations of the 4 desoxynucleoside triphosphates in the synthetic reaction. Similar results were observed with markedly distorted substrate concentrations. In the experiment summarized in TABLE 5, the initial concentration of dTTP or of both dTTP and dATP was lowered to one fifth the level of the other triphosphates. As the reaction proceeded, the disparity in relative concentrations was exaggerated and, presumably, the extent of DNA synthesis was curtailed by the exhaustion of the limiting substrate. Nevertheless, the product synthesized maintained the same A+T/G+C ratio as the primer, and the purine content was equivalent to the pyrimidine content. Finally, the base ratios of

the synthesized product were found to be essentially the same regardless of whether it was isolated early in the reaction or after synthesis had stopped. To determine whether this result held true even at the earliest stages of reaction,

TABLE 4

PURINE AND PYRIMIDINE COMPOSITION OF ENZYMATICALLY SYNTHESIZED DNA*

DNA	No. of analyses	A	T	G	C	$\frac{A+T}{G+C}$	$\frac{A+G}{T+C}$
M. phlei							
Primer	3	0.65	0.66	1.35	1.34	0.49 (0.48–0.49)	1.01 (0.98–1.04)
Product	3	0.66	0.80	1.17	1.34	0.59 (0.57–0.63)	0.85 (0.78–0.88)
A. aerogenes							
Primer	1	0.90	0.90	1.10	1.10	0.82	1.00
Product	3	1.02	1.00	0.97	1.01	1.03 (0.96–1.13)	0.99 (0.95–1.01)
E. coli							
Primer	2	1.00	0.97	0.98	1.05	0.97 (0.96–0.99)	0.98 (0.97–0.99)
Product	2	1.04	1.00	0.97	0.98	1.02 (0.96–1.07)	1.01 (0.96–1.06)
Calf thymus							
Primer	2	1.14	1.05	0.90	0.85	1.25 (1.24–1.26)	1.05 (1.03–1.08)
Product	6	1.19	1.19	0.81	0.83	1.46 (1.22–1.67)	0.99 (0.82–1.04)
T2 bacteriophage							
Primer	2	1.31	1.32	0.67	0.70	1.92 (1.86–1.97)	0.98 (0.95–1.01)
Product	2	1.33	1.29	0.69	0.70	1.90 (1.82–1.98)	1.02 (1.01–1.03)
Synthetic A-T copolymer	1	1.99	1.93	<0.05	<0.05	>40	1.05

* The reaction mixtures and the procedure for isolation of the products were the same as those described for Experiments 2 to 5, TABLE 1. A, T, G, and C refer to adenine, thymine, guanine and cytosine, respectively, except that C in the case of T_2 bacteriophage primer refers to hydroxymethylcytosine. The values given represent averages of the number of analyses indicated. The figures in parentheses are the ranges of values obtained.

TABLE 5

EFFECT OF RELATIVE SUBSTRATE CONCENTRATIONS ON COMPOSITION OF SYNTHETIC DNA

Substrates (relative molar concentration)				Net synthesis	Products	
dCTP	dGTP	dTTP	dATP		$\frac{A+G}{C+T}$	$\frac{A+T}{G+C}$
1.0	1.0	1.0	1.0	11×	1.00	1.98
1.0	1.0	0.2	1.0	6×	1.04	1.82
1.0	1.0	0.2	0.2	6×	0.97	1.82
T_2 bacteriophage DNA as primer					0.98	1.92

isotopically marked substrates were used to distinguish the newly synthesized DNA. In such an experiment, 2 identical reaction mixtures were set up, 1 containing C^{14}-dCTP and the other C^{14}-dTTP. The amount of enzyme and the length of incubation were adjusted to permit an increase of DNA of as little as 2 per cent relative to the DNA added as primer. After incubation, aliquots were removed and assayed for incorporation of isotope into DNA. As shown in TABLE 6, the T/C ratio of the product was similar to that of the primer when

net increases were small (2 to 63 per cent), or large (>1000 per cent, as in TABLE 4).

From these findings, as well as from the observations on the specific replacement of the naturally occurring desoxynucleotide substrates by related analogues, it is clear that the equivalence of adenine to thymine and guanine to cytosine is an inherent feature of DNA synthesis by the "polymerase" enzyme of *E. coli*. Furthermore, the data suggest that the DNA added to the reaction is serving as a template for the enzymatic replication of DNA.

TABLE 6

BASE RATIOS OF THE PRODUCT ISOLATED EARLY IN THE REACTION AS DETERMINED WITH ISOTOPICALLY MARKED SUBSTRATES*

Primer DNA	Increase in DNA, per cent	T incorporated, mμmoles	C incorporated, mμmoles	T/C ratios	
				Product	Primer
M. phlei	2	0.047	0.11	0.42	0.49
M. phlei	35	0.97	2.05	0.47	
Calf thymus	8	0.40	0.28	1.43	1.24
Calf thymus	63	3.08	2.43	1.27	
A. aerogenes	18	0.74	0.93	0.79	0.82

* Duplicate reaction mixtures, as described in TABLE 1 for experiments 2 to 5, were used in each experiment with the exception that C^{14}-dTTP was used in 1 vessel and C^{14}-dCTP was used in the second. After the appropriate incubation period, aliquots were removed and incorporation of isotope into DNA was determined as reported for assay of polymerase.[2]

TABLE 7

ENZYMATIC SYNTHESIS OF VISCOUS DNA FROM NONVISCOUS PRIMERS

	Thymus DNA	Heated thymus DNA	ΦX174 DNA
Reaction:			
Rate	1×	2×	2×
Extent	~ tenfold	~ tenfold	~ tenfold
Viscosity: (dl./gm.)			
Primer	45		1
Product	41	20	22
Heated product	<1	—	<1
% Hyperchromic effect:			
Primer	30	—	10
Product	30	—	30

In view of the possible participation of the DNA primer as a template in the synthetic reaction, it is pertinent to ask to what extent the size and structure of the DNA primer influence the enzymatically synthesized product. It has already been reported that extensive degradation of DNA by the action of pancreatic desoxyribonuclease or dilute acid destroys its priming capacity.[3, 11] On the other hand, heat treatment (100° C. for 10 min.) of calf thymus DNA, resulting in collapse of its macromolecular structure as evidenced by a 30 per cent hyperchromicity and loss of viscosity, produces a primer that supports DNA synthesis at twice the rate and to the same extent as the unheated material (TABLE 7); moreover, a viscous product results. Essentially analogous results were observed using the low molecular weight and comparatively

nonviscous DNA isolated by Sinsheimer from the small phage ΦX174.[12] Again, the synthetic rate was approximately twice that observed with calf thymus DNA and the extent of synthesis was 10 times that of the added primer. Starting with a primer with reduced viscosity of the order of 1 dl./gm., a product was synthesized with a viscosity more than twentyfold that of its primer. Such a product responded to heating or the action of pancreatic desoxyribonuclease in a manner quite comparable to native calf thymus DNA. Thus, upon heating, the viscosity of the product dropped from a value of 22 to less than 1 (grams per 100 ml.)$^{-1}$ and, upon treatment with pancreatic desoxyribonuclease, a 30 per cent hyperchromic effect was observed, as opposed to the 10 per cent hyperchromicity characteristic of the ΦX174 DNA primer.

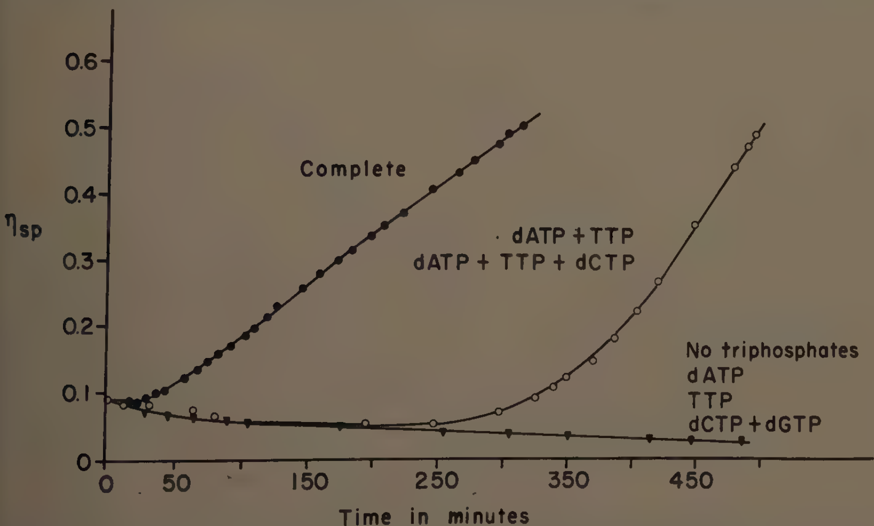

FIGURE 4. Synthesis of DNA and of desoxyadenylate-thymidylate copolymer, with thymus DNA as primer.

It is clear from these results that a collapsed DNA molecule is a highly efficient primer for DNA synthesis by the *E. coli* polymerase and will initiate synthesis of the organized, hydrogen-bonded structure characteristic of most native DNAs. Of further interest in this regard is the observation already noted[3] that treatment of a primer with minute amounts of crystalline pancreatic desoxyribonuclease

$$\left(\frac{\mu\text{g. DNA}}{\mu\text{g. desoxyribonuclease}} = 2 \times 10^5\right)$$

results in a two- to threefold stimulation in synthetic rate. The decision, however, as to whether disruption, or at least some modification, of the macromolecular organization of the primer is essential for DNA synthesis in this system must await an enzyme preparation that is completely free of desoxyribonuclease activity.

To return to a consideration of the desoxyadenylate-thymidylate (A-T) copolymer briefly referred to earlier in the paper: as shown in FIGURE 4, a

viscous polymer was formed in the presence of dATP and dTTP after a lag period of about 4 hours. A similar polymer is formed even in the absence of added DNA. Measurements of polymer synthesis during this interval (by conversion of radioactive dATP or dTTP into an acid-insoluble form) demonstrated a comparable lag. We have not observed formation of the corresponding desoxyguanylate-desoxycytidylate (G-C) copolymer. Once formed and isolated, the A-T polymer can initiate the synthesis of a new polymer with no time lag (FIGURE 5). As shown in TABLE 4, the A-T polymer consists exclusively of desoxyadenylate and thymidylate despite the presence during synthesis of all 4 of the desoxynucleoside triphosphates. Physical examination of such polymers has shown that they have the sedimentation properties and viscosity characteristic of native DNA.

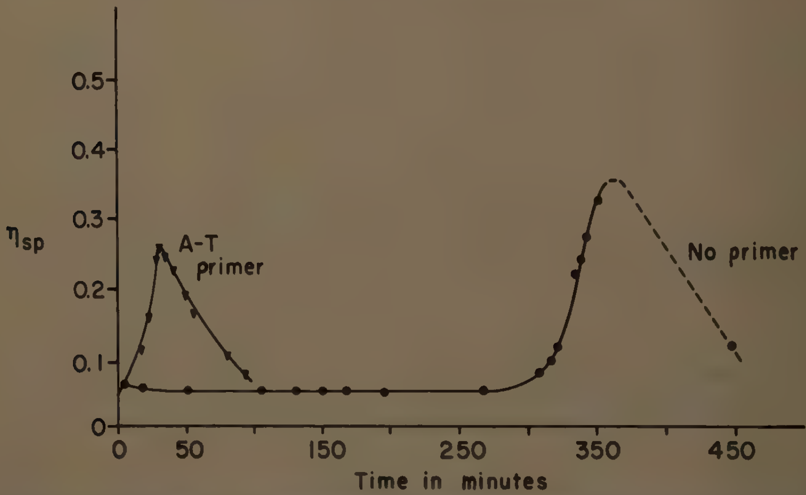

FIGURE 5. Synthesis of desoxyadenylate-thymidylate copolymer in the presence and absence of an A-T primer.

A unique feature of the A-T polymer is its extreme responsiveness to desoxyribonuclease activity. Thus, once synthesis of the polymer has ceased, it is very rapidly degraded to a nonviscous (and acid-soluble) form by the nuclease activity present in the polymerase preparation (FIGURE 5). It is conceivable that our failure to observe synthesis of a G-C polymer is merely a manifestation of the even greater responsiveness of such a polymer to nuclease action.

Perhaps one of the most interesting and puzzling features of this reaction is the significance of the prolonged lag period that precedes initiation of synthesis. One might speculate that it is during this interval that the appropriate primer is somehow developed. Although no acid-insoluble materials can be detected during this period, a search for small polynucleotides might well prove fruitful.

Summary

Physical characterization of the DNA synthesized by the *E. coli* polymerase in the presence of 4 desoxynucleoside triphosphates and a DNA primer has

shown it to have the basic structural features commonly associated with DNA carefully isolated from natural sources. It has a sedimentation coefficient of approximately 25 S and a reduced viscosity ranging from 15 to 45 dl./gm. A 30 per cent hyperchromic effect results upon treatment with pancreatic desoxyribonuclease.

Chemical analysis of the enzymatically synthesized DNA shows it to possess the equivalence of adenine to thymine and guanine to cytosine ratios required by the Watson-Crick model for DNA. In confirmation of this result is the finding that the desoxynucleoside triphosphates of the purines and pyrimidines that occur naturally in DNA can be replaced specifically in the enzymatic reaction only by those analogues whose hydrogen-bonding capacities are in accord with the Watson-Crick model. The ratio of A+T/G+C in the enzymatic product is very close to that of the primer DNA used in its synthesis; primer values have ranged from 0.50 to greater than 40. The values obtained are independent of the relative concentrations of the desoxynucleotide substrates used in the synthesis, or of the extent of net synthesis. These results have led to the hypothesis that the DNA primer participates in the synthetic reaction directly as a template.

DNA that has undergone collapse of its secondary structure as a result of heating, as well as the DNA of relatively small molecular weight from the small phage ΦX174 will support efficient synthesis of a viscous, hypochromic product.

The *E. coli* polymerase is capable, after a time lag of 3 to 6 hours, of synthesizing, in the absence of added DNA, a viscous DNA-like copolymer of desoxyadenylate and thymidylate. Such a polymer, in turn, can initiate, with no lag, synthesis of a new polymer and, despite the presence of all 4 desoxynucleotide substrates in the reaction, will contain exclusively desoxyadenylate and thymidylate.

Acknowledgments

This article is a result of the joint efforts of Arthur Kornberg, to whom I am gratefully indebted for having introduced me to the chemistry and enzymology of the nucleic acids and for his guidance and stimulation throughout this investigation, Maurice Bessman, Howard Schachman, Julius Adler, Steven Zimmerman, and Ernest Simms. I wish to thank Robert Sinsheimer for his gift of a ΦX174 DNA preparation.

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STUDIES ON THE CHEMICAL SYNTHESIS AND ENZYMATIC DEGRADATION OF DESOXYRIBO-OLIGONUCLEOTIDES*

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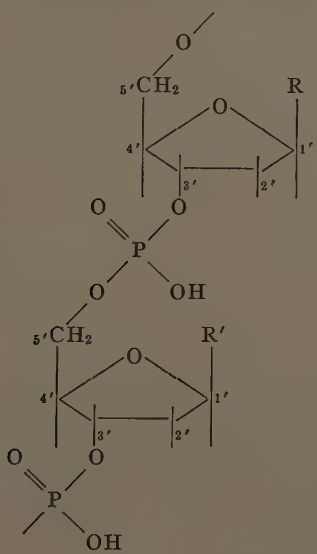
British Columbia Research Council, Vancouver, B.C., Canada

The last decade has seen remarkable progress in the chemistry of both the ribo- and desoxyribonucleic acids. It is now known with certainty that in both types of nucleic acids the individual nucleosides are joined through phosphodiester bonds that link the 3′-hydroxyl group of the sugar moiety in 1 nucleoside to the 5′-hydroxyl group of the next nucleoside, as shown below.



I

The problems to be undertaken next in connection with the chemistry of these biologically important macromolecules are (1) synthesis of polynucleotides containing specifically the naturally occurring $C_{3'} \rightarrow C_{5'}$ internucleotidic linkage; (2) development of methods for the separation of polynucleotides; and (3) methods for the determination of the nucleotide end groups and sequences in polynucleotide chains. In our laboratory we initiated studies on various aspects of these problems, and this paper summarizes our recent work on (1) a method for the synthesis of $C_{3'} \rightarrow C_{5'}$ internucleotidic linkage and its application to the stepwise synthesis of mixed oligonucleotides; (2) synthesis of polynucleotides by chemical polymerization of mononucleotides; and (3) elucidation of the mode of action of snake venom and spleen phosphodiesterases, using the chemically synthesized substrates. Studies on the mode of action of phosphodiesterases and nucleases are important not only because they are likely to

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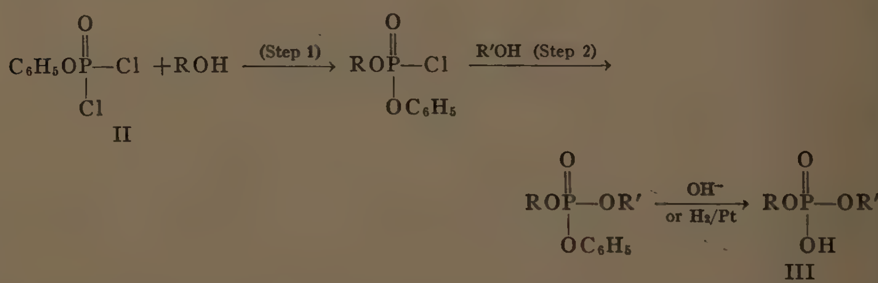
shed light on the role of such enzymes in polynucleotide metabolism but also because, from the practical standpoint, such enzymes offer potentially powerful tools in structural analysis of polynucleotides. However, in order that these enzymes may be used for structural analysis it is essential to determine precisely their specificities and mode of action, and this is possible only when substrates of completely defined structure are available.

The Synthesis of Internucleotidic Linkage

The first major requirement in synthetic analysis of polynucleotides is a satisfactory method for the formation of unsymmetrical phosphodiesters of the general structure (III). Two methods have been introduced for this purpose; the first utilizes the bifunctional phosphorylating agent (II) in reactions illustrated by Scheme 1.

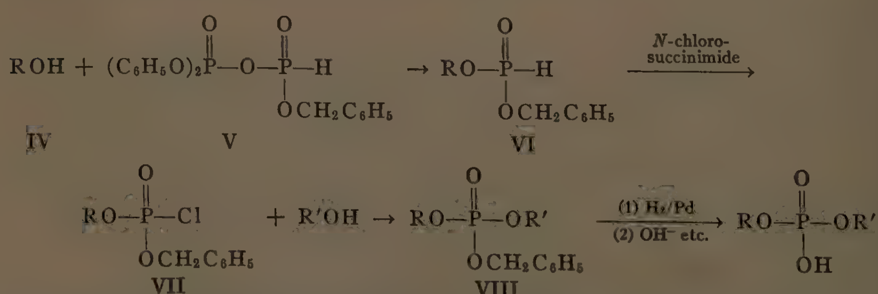
Scheme 1:



This method has been used successfully in the extensive work on synthesis in the phospholipid field by Baer and Stancer.[1] Attempts in our laboratory to use an analogous procedure for the synthesis of internucleotidic linkages gave good results for preparing $C_{5'} \rightarrow C_{5'}$-linked dinucleoside phosphates, but were unsuccessful in forming $C_{3'} \rightarrow C_{5'}$ bonds, presumably because of the relatively unreactive $C_{3'}$-hydroxyl group.

The second method previously reported is that used by Michelson and Todd[2] in the synthesis of dithymidine dinucleotide, the only dinucleotide (containing the $C_{3'} \rightarrow C_{5'}$ linkage) synthesized by chemical means prior to the present work. The principle of this method is illustrated as follows:

Scheme 2:

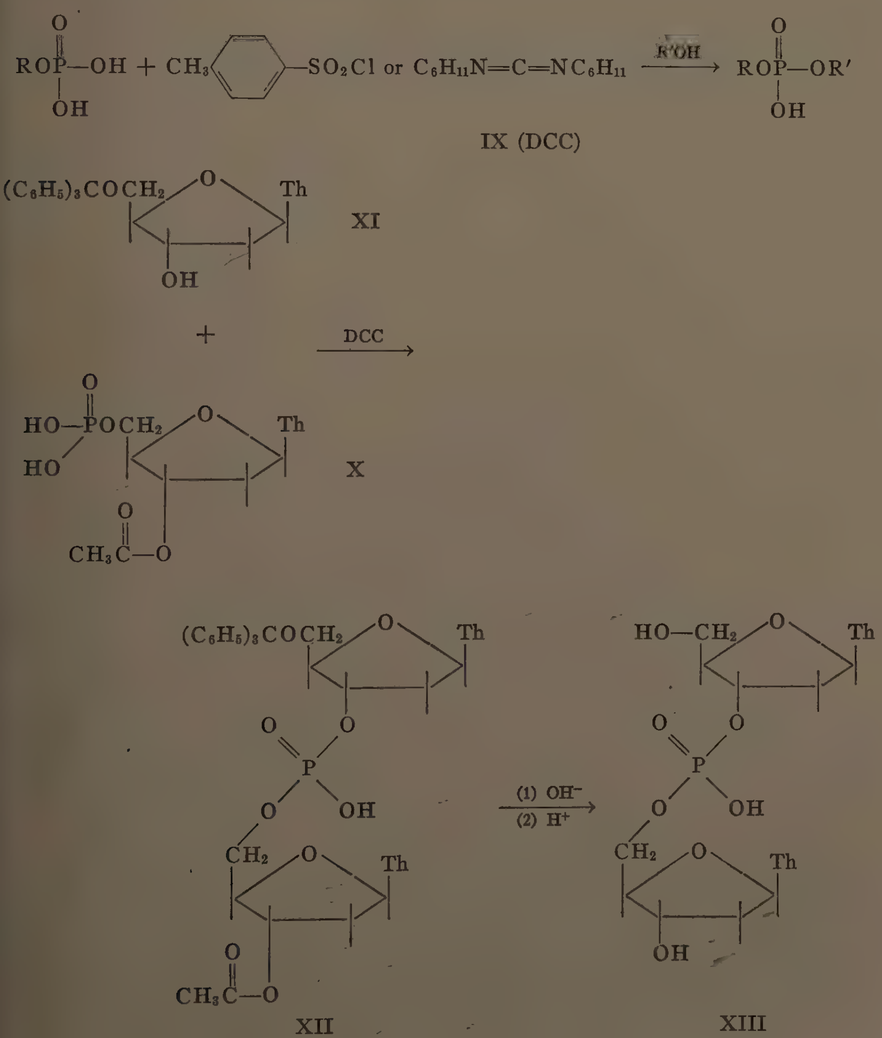


An appropriately blocked nucleoside (IV) is phosphorylated by the reagent (V) to give the nucleoside benzyl phosphite (VI). This neutral substance is con-

verted to the phosphorochloridate (VII) by treatment with *N*-chlorosuccinimide. The phosphorochloridate is then used to phosphorylate the second suitably protected nucleoside, to give the neutral ester (VIII). Removal of the benzyl and other protecting groups gives the desired dinucleoside phosphate. The yields by this procedure were low and, because of the problems involved in purification of the neutral intermediates, it is doubtful if it could be extended to higher polymers.

The approach we have developed for the synthesis of phosphodiester linkages involves the *direct* activation of a monoalkyl phosphate with either *p*-toluenesulfonyl chloride[3a] or, preferably, dicyclohexyl carbodiimide[3b] (IX) and reaction with the hydroxyl group of a second molecule according to Scheme 3:

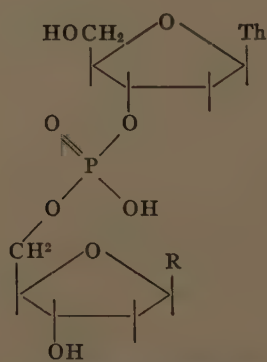
Scheme 3:



In general, high yields of the desired diesters are obtained, and the advantages of using monoalkylesters (mononucleotides) directly in the synthesis are obvious.

In the initial study of the above method,[4] the reaction of 3′-*O*-acetyl thymidine-5′ phosphate (X) with another protected nucleoside, 5′-*O*-triphenylmethyl (trityl) thymidine (XI), was carefully examined. The yield of dithymidine phosphate (XIII) isolated after mild alkaline and acidic treatments of the initial product (XII) was optimum after about 2 days and was more than 60 per cent of theoretical; the rest of the materials were recovered as the mononucleotide and as thymidine.

The method was next applied to the synthesis of mixed dinucleoside phosphates. The two compounds (XIV and XV) were again obtained in good yield by using, respectively, *N*,*O*-diacetyldesoxyadenosine-5′ phosphate and *N*,*O*-diacetyldesoxycytidine-5′ phosphate as the "nucleotide" components in the above synthesis.



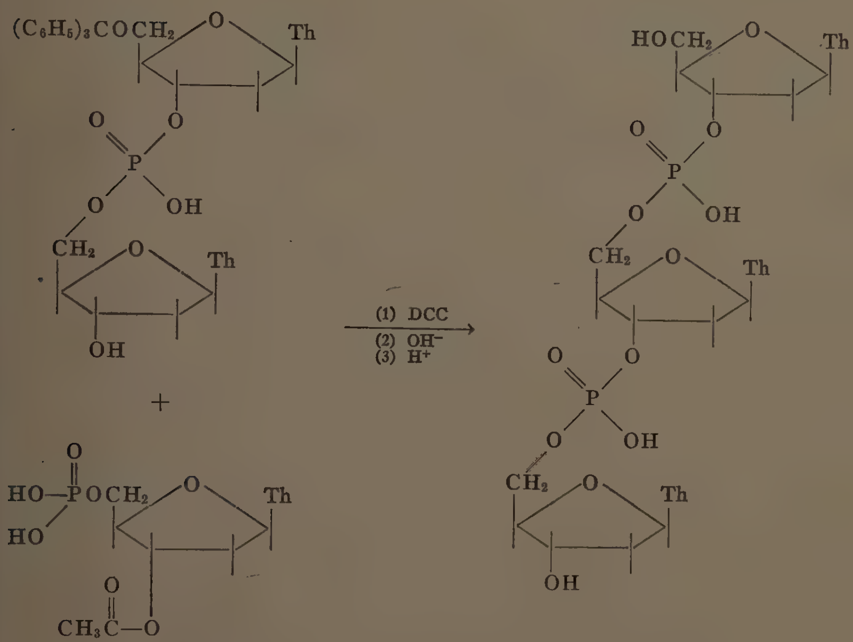
XIV (where R is adenine)
XV (where R is cytosine)

Stepwise Synthesis of Mixed Oligonucleotides

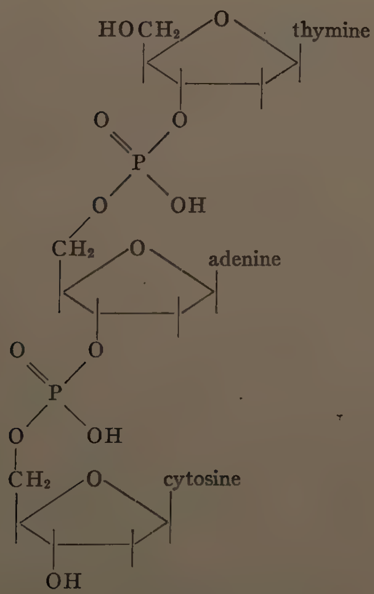
The stepwise synthesis of oligonucleotides containing different mononucleotide units requires the selective unblocking of an hydroxyl function at one end of the fully protected dinucleoside phosphates, followed by condensation with a suitably protected nucleotide. Mild alkaline treatment of the intermediate (XII) obtained above gave a product (XVI) that was brought into reaction with 2 molar equivalents of 3′-*O*-acetyl thymidine-5′ phosphate. After removal of the protecting groups (Scheme 4), trithymidine diphosphate (XVII) was obtained in about 60 per cent yield.

This method was then investigated for the synthesis of mixed trinucleoside diphosphates, and the synthesis of desoxycytidylyl-(5′ → 3′)-desoxyadenylyl-(5′ → 3′)-thymidine (XVIII) was realized. The problems introduced in this synthesis by the much greater lability of purine glycosyl bonds and the reactivity of the ring amino groups are discussed elsewhere.[6] Extension of this work to the synthesis of higher oligonucleotides is in progress.

Scheme 4:



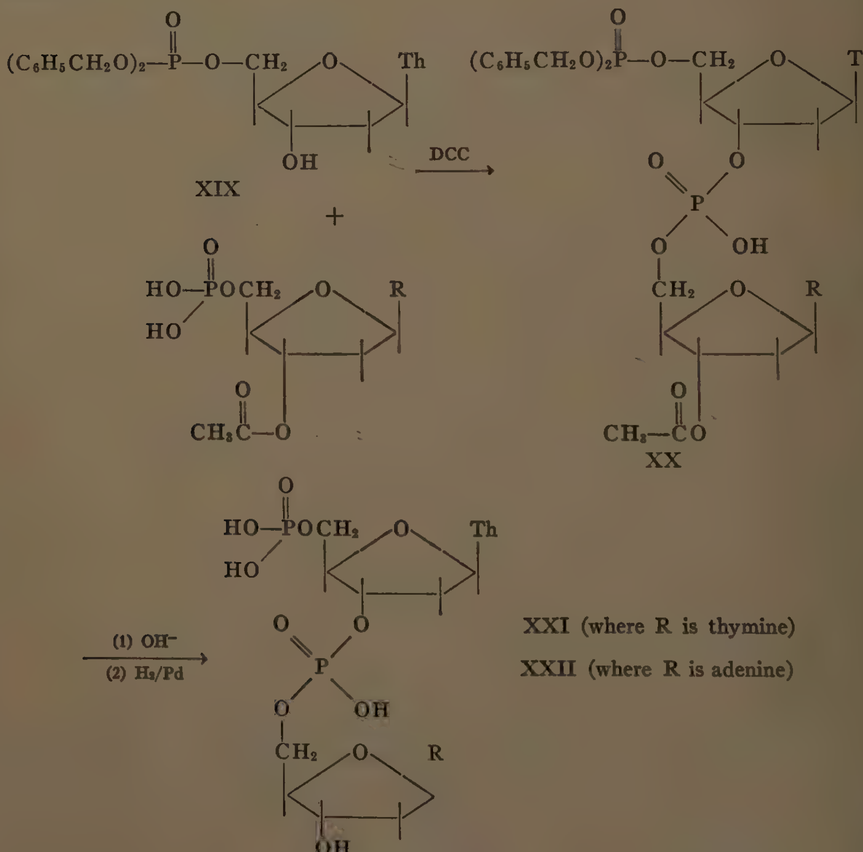
XVI XVII



XVIII

Linear oligonucleotides usually carry a phosphomonoester group at one or the other end of the chain, and the above synthetic methods can be adapted to yield such "true" oligonucleotides. Two basic approaches have been used. The first is that in which a protected phosphoryl group is used as a blocking group on a nucleoside at the outset of the synthesis. The synthesis of 2 dinucleotides bearing 5′-phosphate end groups (XXI, XXII) by this method is illustrated below.

Scheme 5:



XXI (where R is thymine)

XXII (where R is adenine)

In the second approach, the phosphomonoester end group is introduced after the synthesis of the internucleotidic linkages. Thus, for example, mild acidic treatment of the fully protected dinucleosidephosphate (XII) selectively exposes the 5′-hydroxyl group (XXIII), and subsequent phosphorylation with dibenzyl phosphorochloridate and removal of the protecting groups gives the dinucleotide (XXI) as shown in Scheme 6.

Synthesis of Oligonucleotides by Polymerization of Mononucleotides

In the above method of synthesis it was to be expected that mononucleotides bearing unsubstituted 3′-hydroxyl groups would "self-condense" on treat-

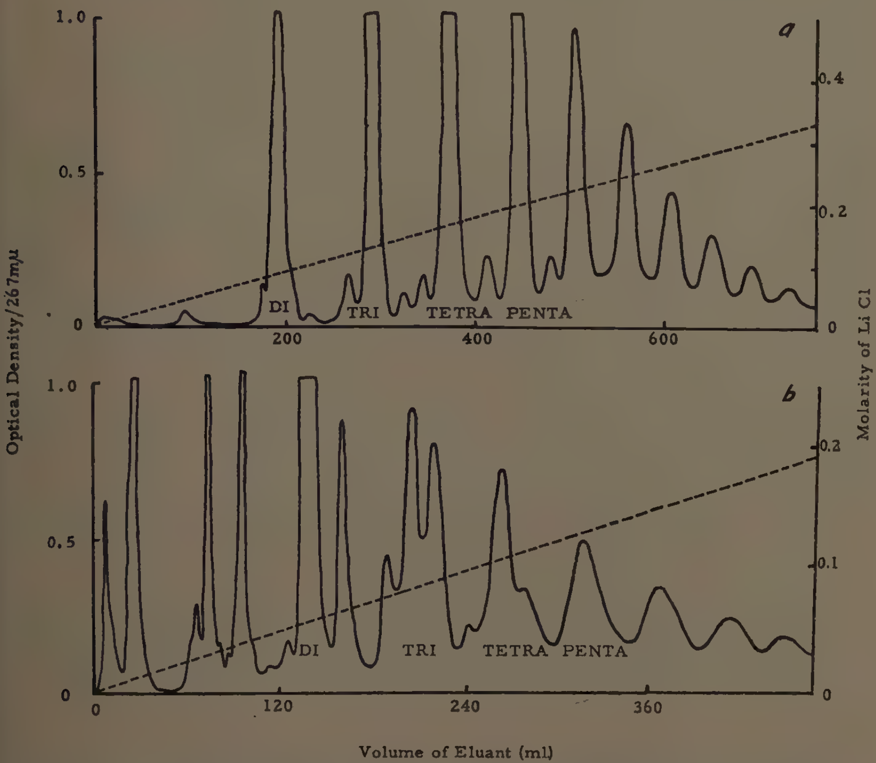
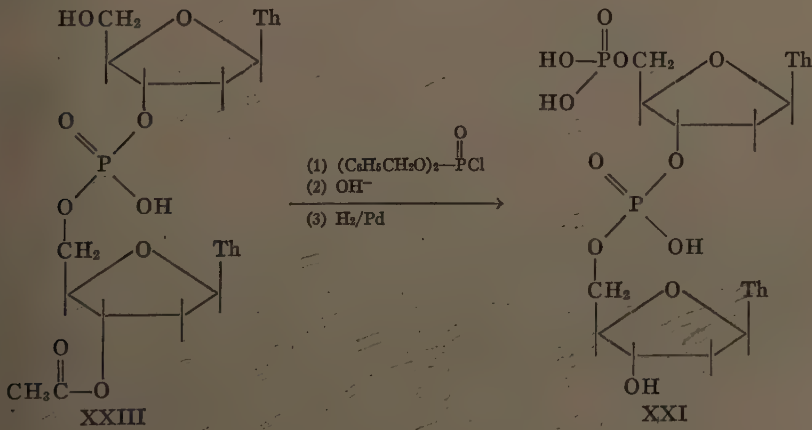


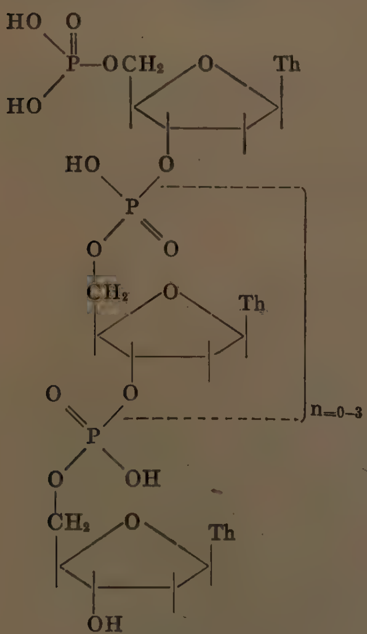
FIGURE 1. Chromatographic analysis of thymidine oligonucleotides. (*a*) Products of reaction of thymidine-5′ phosphate with *p*-toluenesulfonyl chloride. The total mixture was dialyzed against distilled water, and 220 optical density units (267 mμ) of the nondialyzable material was chromatographed on a DEAE-cellulose column (16.5 × 0.9 cm.) using linear salt (lithium chloride) gradient as shown by the broken line. Flow rate is about 1.2 ml./min. (*b*) Total products of reaction of thymidine-5′ phosphate with DCC chromatographed on a ECTEOLA-cellulose column (8 × 0.9 cm.) using linear salt gradient as shown by broken line. Flow rate is 0.7 ml./min.

Scheme 6:

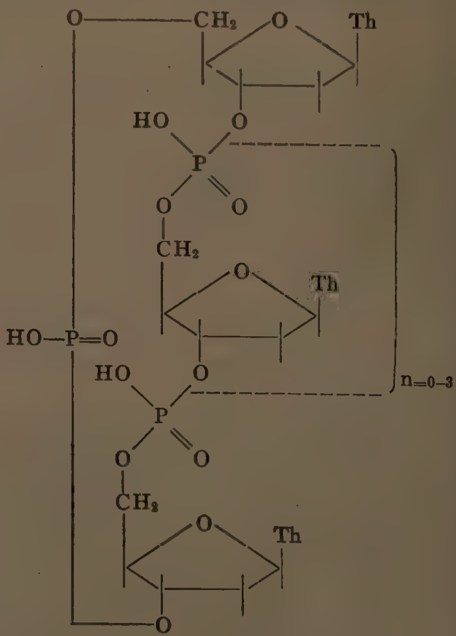


ment with the activating agents (*p*-toluene sulfonyl chloride, or DCC) to form polymeric products. The reaction was first studied on the simplest nucleotide thymidine-5′ phosphate, and polymers were indeed obtained.[6] In attempts to separate the mixture, paper chromatography was used initially with partial success. Anion-exchange resin columns used in the past for separation of oligonucleotides were unsatisfactory in the present work. Anion exchangers that proved strikingly useful in the separation of the mixtures are the amino alkyl–substituted celluloses developed by Peterson and Sober.[7] Both DEAE and ECTEOLA cellulose have been used, but the latter is currently used exclusively because of its superior flow characteristics and reproducibility. Elution diagrams of 2 such separations, carried out using the linear salt-gradient technique, are shown in FIGURE 1. FIGURE 1*b* shows the results of analysis of a total reaction mixture, while FIGURE 1*a* is an analysis after dialysis of the mixture against distilled water, which removes mononucleotidic materials predominantly, and some dinucleotidic. Attention has been focused initially on the first major peaks at the left in FIGURE 1, and members up to the penta-nucleotide level have been isolated pure and characterized. Extrapolation of the present data shows that polynucleotides containing up to 11 thymidylic acid residues are present in the mixtures.

The major peaks can be fractionated further either on the columns or, subsequently, on paper sheets to give two series of compounds—the first being the *linear* and the second the *cyclic* oligonucleotides. The general structures of



XXIV

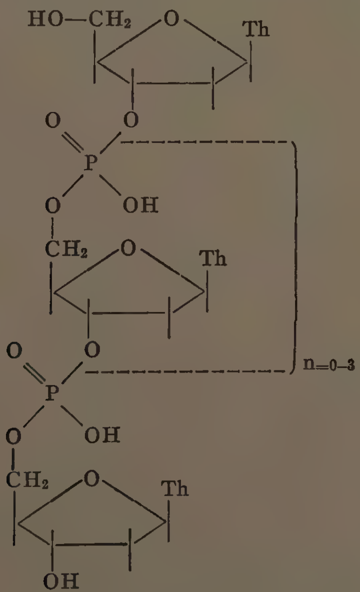


XXV

these two homologous series of compounds are shown below (XXIV and XXV, respectively).

The linear oligonucleotides contain a 5′-phosphomonester group at one end and a 3′-hydroxyl group at the other end and have the repeating, naturally occurring (5′ → 3′), internucleotidic linkage. They were characterized in a number of ways: the homogeneity of each member was established by paper chromatography in 3 solvent systems and by paper electrophoreses under acidic, neutral, and alkaline conditions. The ratio of phosphorus to thymidine was close to 1 for all the members. Purified snake venom diesterase degraded them to thymidine-5′ phosphate, thus supporting the conclusion that the compounds were simple polymers of the mononucleotide. Members of this series were found by potentiometric titrations to have the expected ratios of primary to secondary phosphoryl dissociations and were shown by acetylation to contain the end 3′-hydroxyl function.

Prostatic phosphomonoesterase removed the phosphomonoester groups from the linear oligonucleotides, and the resulting products, whose general structure is shown below (XXVI), were then degraded by snake venom and spleen diesterases. The results, which are discussed in detail, subsequently confirmed the structures of these products.

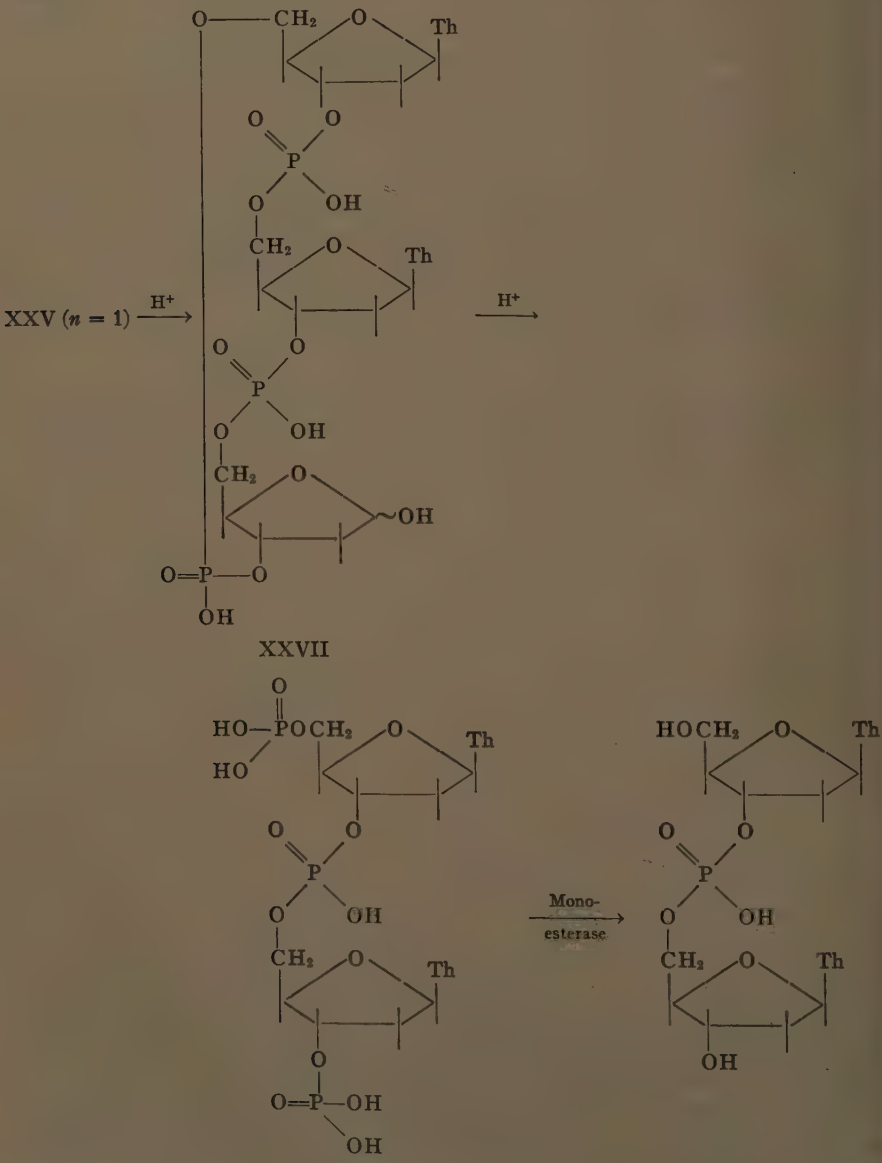


XXVI

The cyclic oligonucleotides (XXV) arise from the end-to-end cyclization of the linear oligonucleotides (XXIV). The extent of this cyclization process, which competes with linear polymerization, decreases with increase in chain length. Thus, while at the dinucleotide level, the cyclic member is much more abundant than the linear dinucleotide, at the pentanucleotide stage the cyclic compound forms only a small portion of the total fraction. The assigned

structures for this homologous series of compounds were shown in a variety of ways and only a brief summary of the total evidence is presented. Thus, they lacked both the phosphomonoester end groups and the 3′-hydroxyl functions. That they represented a homologous series was clear from their mobilities on paper chromatograms and from the fact they they had the same relative electrophoretic mobilities under acidic, neutral, and alkaline conditions. Their

Scheme 7:



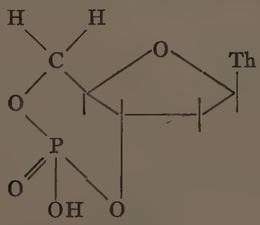
XXVII

XXVIII

order of elution from columns enabled correlation of their size with the linear members. Paper-electrophoretic mobility of each cyclic homologue was identical with that of the corresponding linear compound under acidic conditions, but lower, as expected, under neutral conditions.

A degradative technique, based on acidic hydrolysis as the first step, made it possible to correlate further the size of the cyclic oligonucleotides. This is illustrated for the cyclic trinucleotide (XXV) in Scheme 7 where $n = 1$.

The initial step during acidic degradation of this symmetrical compound is cleavage of 1 of the glycosyl bonds; the resulting product (XXVII) will suffer rapid degradation at points linking the phosphoryl groups to the reducing desoxyribose fragment, to give dithymidine triphosphate (XXVIII). Further degradation of this product would occur, of course, in an analogous manner, but the aim in the present technique was partially to hydrolyze the cyclic oligonucleotide so as to isolate the largest linear product of degradation—namely, the product containing all the phosphorus of the original molecule, but 1 less thymidine unit. Compounds of the type T_nP_{n+1} were then treated with prostate phosphomonoesterase to remove the phosphomonoester end groups, and the resulting compounds (XXVI) were identified by comparison with the same series of products obtained from the linear oligonucleotides (XXIV) by phosphomonoesterase treatment. In the case of dithymidine triphosphate (XXVIII), the product after dephosphorylation was identical with a synthetic sample of dithymidine monophosphate. As expected, the cyclic tetranucleotide gave trithymidine diphosphate as the major product and, similarly, the cyclic pentanucleotide gave tetrathymidine triphosphate.



XXIX

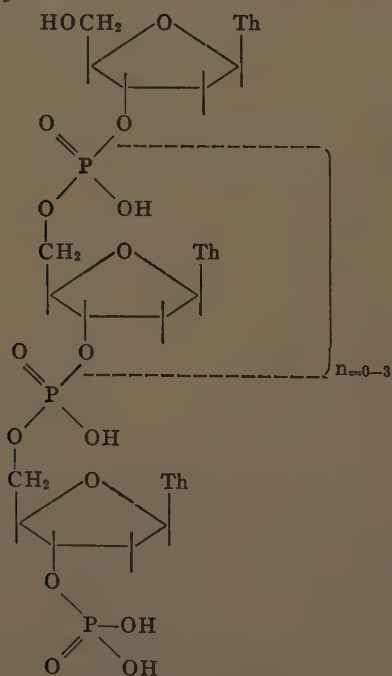
Digression may be made at this point to mention a minor product formed in the polymerization experiments. The substance, which has been shown to be thymidine-3′,5′ cyclic phosphate (XXIX), may in fact be regarded as the "monomeric" member of the cyclic oligonucleotides. Alternative and satisfactory syntheses of members of this class of compounds (ribo- as well as desoxyribonucleoside-3′, 5′ cyclic phosphates) have been devised. It is very significant that a member of this group, adenosine-3′,5′ cyclic phosphate,[8] has been isolated from tissues and has been shown to stimulate the formation of liver glycogen phosphorylase.

Further Polymerization Studies

The studies reported above represent only the initial phase of our program of research on the chemical polymerization of mononucleotides. Some of the obvious and important questions to be asked next are: What are the kinetics

of the above reaction, and how far can chemical polymerization be induced to go? What is the precise mechanism of the reaction, and can alternative and better activating agents be found? Can the polymerization of other mononucleotides (desoxyribo- as well as ribomononucleotides) and mixtures of mononucleotides be realized so as to give a wide range of simple and mixed polynucleotides?

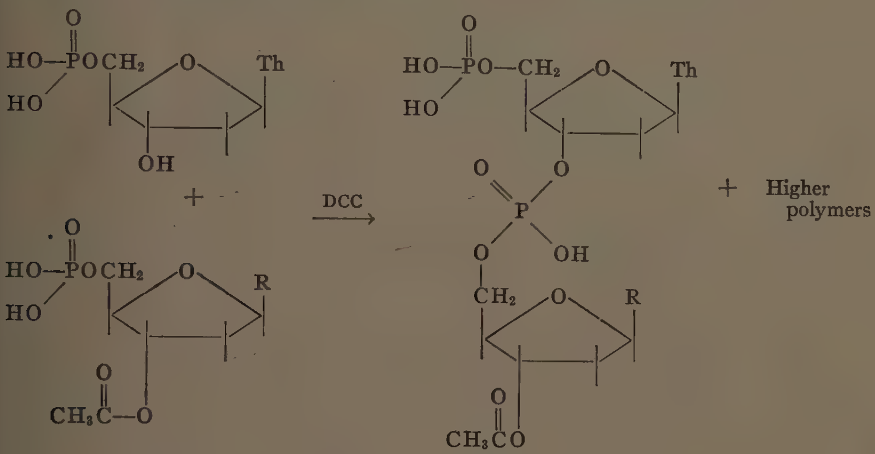
At present we can mention briefly the following extensions of the work described above. Thymidine-3′ phosphate has been synthesized in quantity by a new and improved method and its polymerization has been accomplished,[9] giving homologous series of linear thymidine oligonucleotides that bear 3′-phosphoryl end groups of the general structure shown below (XXX) and giving the corresponding cyclic oligonucleotides identical, as expected, with the compounds described above. The series of oligonucleotides terminated in 3′-phosphomonoester end groups are required for studies of a variety of enzymes such as spleen phosphodiesterase and the group of desoxyribonucleases that degrade desoxyribonucleic acid (DNA) to give smaller fragments carrying 3′-phosphate end groups.



XXX

The cyclic polynucleotides described above, although interesting, are at present not as important for various studies as the linear series. A procedure that greatly reduces the formation of cyclic compounds has been devised. This involves adding 3′-*O*-acetylthymidylic acid to the unprotected thymidylic acid. The protected nucleotide can form only the terminating unit of linear polymers,

and the compounds (XXXI and higher polymers) thus formed are unable to undergo the end-to-end cyclization. The acetyl groups can be removed readily at the end by mild alkaline treatment.



XXXI (where R is thymine)
XXXII (where R is cytosine)

By copolymerizing 2 equivalents of thymidylic acid with 1 of 3′-*O*-acetyl derivative, 4 times more linear dinucleotide than cyclic dinucleotide was produced. The ratio was reversed when thymidylic acid alone was polymerized. The yields of the higher linear polynucleotides during the above copolymerization were even more favorable.

The copolymerization of one protected nucleotide with another "free" nucleotide can give very useful series of compounds. Thus, by copolymerizing *O,N*-diacetyl desoxycytidylic acid and thymidylic acid a series of linear thymidylic acid polymers, which are all terminated in a desoxycytidylic residue, are obtained (XXXII).

The Mode of Action of Snake Venom and Spleen Phosphodiesterases

Snake venom phosphodiesterase has served in many previous studies as a useful tool for degrading RNA and DNA fragments to 5′-mononucleotides.[10, 11] In contrast, a spleen phosphodiesterase, recently highly purified by Hilmoe,[12] has been shown by Heppel and his co-workers[13] to degrade ribo-oligonucleotides to ribonucleoside-3′ phosphates. Using the synthetic oligonucleotides described above, it has now proved possible to obtain further insight into the mode of action of these 2 enzymes.[14, 15] The series of compounds first studied were the linear thymidine oligonucleotides from which the phosphomonoester end groups had been removed by treatment with phosphomonoesterase (XXVI). The noteworthy feature of these substances, from the present standpoint, is that they all possess a free 3′-hydroxyl group at 1 end and a 5′-hydroxyl group at the other. The results of a kinetic study of the action of venom diesterase on pentathymidine tetraphosphate T_5P_4 (XXVI), where $n = 3$, are shown in

FIGURE 2. The data clearly show that the degradation proceeds stepwise from the end of the chain bearing the 3′-hydroxyl group; attack on the terminal phosphodiester bonds liberates thymidine-5′ phosphate and the lower homologue. The process continues and thymidine appears only toward the end of degradation.

The paper-chromatographic results of the kinetic study of the degradation of T_5P_4 and its lower homologues by the spleen diesterase are also very similar to those shown for the venom diesterase (FIGURE 2) except for the important difference that, while the mononucleotide that accumulates in venom diesterase

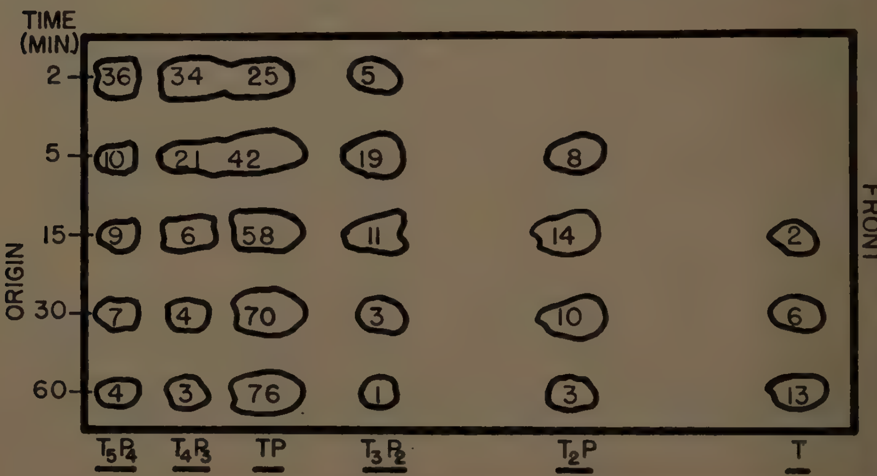


FIGURE 2. Chromatography of venom phosphodiesterase reaction products. The reaction mixture consisted of M trihydroxymethylaminomethane buffer, pH 8.9 (10 μl.), pentathymidine tetraphosphate (14 optical density units measured at 267 mμ), venom diesterase preparation (31 μg. in 7 μl.), and water (20 μl.). Aliquots (6 μl.) were removed at intervals shown, mixed rapidly with 1 μl. of glacial acetic acid, and the mixture applied to Whatman 3-mm. paper. Descending chromatography was carried out using isopropyl alcohol-ammonia-water (7:1:2) overnight. T_5P_4 represents pentathymidine tetraphosphate; T_4P_3, tetrathymidine triphosphate; TP, thymidine 5′-phosphate; T_3P_2, trithymidine diphosphate; T_2P, dithymidine monophosphate; and T, thymidine. The figures in each spot are the percentages of the total optical density in each aliquot.

degradation is thymidine-5′ phosphate, that with the spleen diesterase is thymidine-3′ phosphate. It is concluded, therefore, that the mode of action of spleen diesterase is also stepwise, but complementary to that of the venom diesterase in that it proceeds from the opposite end of the chain—that is, from the end bearing the 5′-hydroxyl group.

The above findings are illustrated in FIGURE 3, where shorthand formulations are used for the oligonucleotides.

The observation that the dinucleotides bearing 5′-phosphate end groups are hydrolyzed by the venom diesterase much faster than are the dinucleoside phosphates (lacking the terminal 5′-phosphate group)* led Laskowski and his

* Likewise, in the case of spleen diesterase, the rate of degradation of oligonucleotides bearing a 3′-phosphate end group is much higher than that of compounds (XXVI) lacking such an end group.

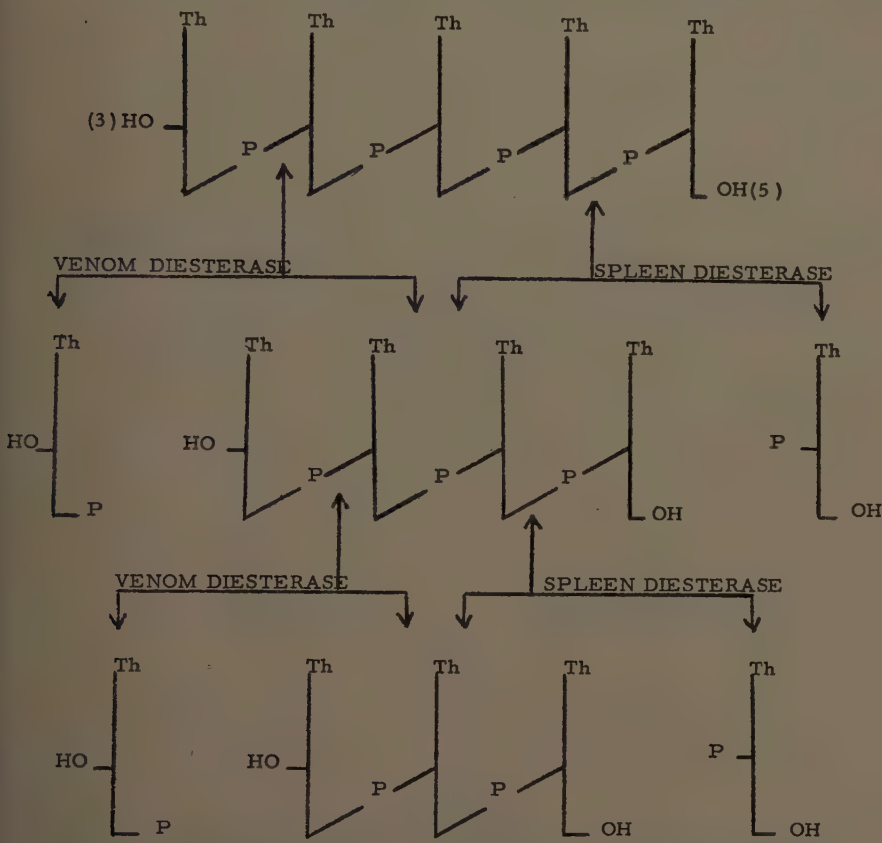


FIGURE 3. Schematic representation of the degradation of thymidine oligonucleotides by snake venom and spleen phosphodiesterases.

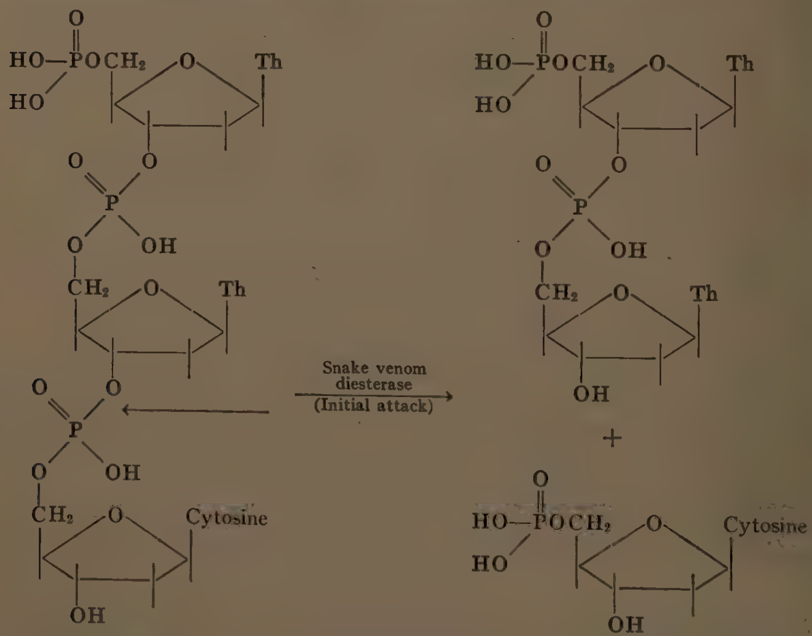
TABLE 1
COMPARATIVE RATES OF HYDROLYSIS OF PHOSPHODIESTERS BY VENOM ENZYME

Substrate	Rate µM/hour/mg.	K_m M/l.
Dithymidine monophosphate (XIII)*	278	5.3×10^{-4}
Thymidylyl-(5′ → 3′)-thymidylic-(5′) acid (XXI)*	6840	2.1×10^{-4}
Thymidine cyclic dinucleotide* (XXV)†	about 80	—
Thymidine-5′ *p*-nitrophenyl phosphate	36,500	5.0×10^{-4}
Bis-*p*-nitrophenyl phosphate	39	7.7×10^{-4}

* Higher homologues in each series are hydrolyzed at similar rates when tested at equimolar concentrations.
† Where n = 0.

co-workers[16] to make the interesting suggestion that the action of the venom was like that of "carboxypeptidase" and that it removed, successively, nucleoside-5′ phosphate units from the end bearing the 5′-phosphate end group. Results of our kinetic studies on a number of related compounds are shown in TABLE 1. The data confirm and extend the results of Laskowski and his co-workers insofar as the influence of the 5′-phosphate end group is concerned. However, the mode of action elucidated above is exactly opposite to that postulated by these authors. That the same mode of degradation holds for the series bearing 5′-phosphate end groups was shown by the following 2 types of experiments.

In one experiment a kinetic study of the degradation of the mixed trinucleotide desoxycytidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidylic-(5′) acid (XXXIII) by the diesterase showed that the mononucleotide released first was desoxycytidine-5′ phosphate and not thymidine-5′ phosphate.



XXXIII

In the other experiment, the end 3′-hydroxyl group of linear tetrathymidylic acid was acetylated and the degradation of the resulting product by the diesterase was then studied. Although substitution of the 3′-hydroxyl group apparently reduced the rate, the first product formed was 3′-*O*-acetyl thymidylic acid, thus showing, again, that the degradation did not begin from the end bearing the 5′-phosphate end group.

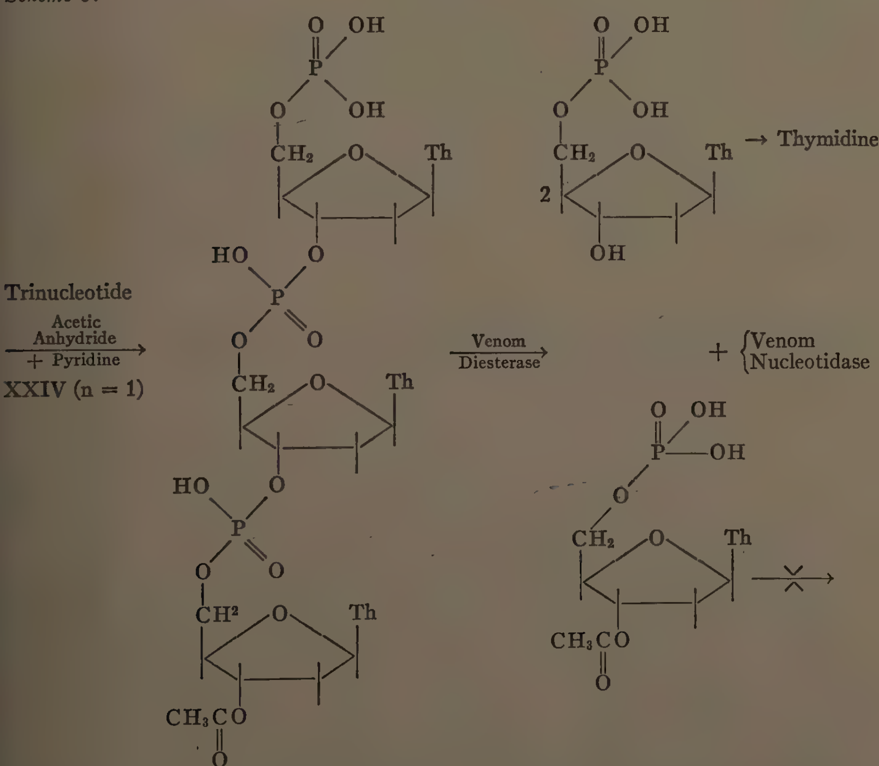
The observations reported above prompt the speculation that the "exo-polynucleotidase" type of action may be quite general for a group of phosphodiesterases; it therefore becomes of immediate interest to study other phosphodiesterases by similar techniques. It may be noted, with regard to the

snake venom and spleen diesterases, that both preparations used in the present work degraded (TABLE 1) the cyclic oligonucleotides (although much more slowly) and that, therefore, the preparations are capable of degrading polynucleotide chains from the interior of the chains.

Acetylation as an End-Group Method

The enzymic results described offer the hope that it will be possible to use the phosphodiesterases for determining the end groups and sequence of nucleotide units in a polynucleotide chain. Chemical methods also are being sought for the end-group analysis of polynucleotides. One promising approach that has been investigated uses thymidine oligonucleotides as models and consists of acetylation of the end 3′-hydroxyl function and subsequent degradation by crude snake venom (Scheme 8). The diesterase first cleaves all the diester bonds, and the mononucleotides thus released are dephosphorylated (by the "5-mononucleotidase" present in the venom) to the corresponding nucleosides.

Scheme 8:



However, the end unit, which is present as the acetylated nucleotide, is not attacked by the mononucleotidase, since this enzyme requires absolutely that the 3′-hydroxyl group be free. It is thus possible, in principle, to determine the end group in a polynucleotide chain. The main problem here appears to

be development of a satisfactory technique for the acetylation of the end hydroxyl group of mixed polynucleotides.

Concluding Remarks

Our present work has led to the development of a method for the synthesis of internucleotidic linkage and, using it, we have already synthesized a number of oligonucleotides. The work of synthesis reported must be regarded strictly as a beginning in a field of vast complexity, and the synthesis of higher mixed polynucleotides still presents a challenge of the highest order to the resources of organic chemistry.

Determination of sequences of nucleotides in polynucleotide chains again poses problems of the utmost complexity. For a long time to come the aim must be to develop chemical, physiochemical, and enzymic methods for structural analysis by using polynucleotides of known structure. The work reported above represents a promising beginning.

In recent years great advances have been made in the determination of sequences of amino acids in polypeptide chains. This in turn has made possible the solution of many complex problems, such as the conversion of the inactive precursors to active enzymes (for example, chymotrypsinogen to chymotrypsin), the nature of the active sites of various enzymes, and the mechanism of their action. All this progress doubtless would not have been possible without the basic work on the synthesis and structure of peptides that was carried out during the last thirty years. With the nucleic acids, too, we may reasonably expect that the progress we make in understanding their biological functions in chemical terms will follow analogous paths.

Acknowledgments

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ENZYMES HYDROLYZING DNA*

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The discovery of new nucleolytic enzymes and progress in understanding the mechanism of action of those already known makes simple division of nucleases into desoxyribonucleases (DNases) and ribonucleases (RNases) inadequate and sometimes misleading.[1] Four criteria for classifying a nuclease are proposed (TABLE 1): the substrates susceptible, the type of attack, the products, and the preferential linkage. The system is arbitrary, and probably it will become necessary to extend the number of independent criteria.

Let us first consider the classic pancreatic DNase I under these headings. DNase I is specific for DNA, it is an endonuclease, and the products are terminated in 5′-phosphate. The location of the preferentially susceptible internucleotide bond will be discussed with respect to bases adjacent to it.

Unfortunately, the present state of our knowledge does not permit us to define "preferential" in terms of the rates at which different internucleotide bonds are hydrolyzed. The word is used in the same sense as in limited proteolysis.

The evidence for preferential splitting comes from the frequency of appearance of dinucleotides of known sequence in digests of DNA by DNase I. The dinucleotides with sequences pPy-pPy,† pPu-pPu, and pPy-pPu were abundant, but those with sequences pPu-pPy were either absent[2] or very rare.[3] This was interpreted to mean that the pPu-pPy bond was preferentially hydrolyzed. Additional evidence was obtained by isolating a trinucleotide ApApTp from a digest of desoxyribonucleic acid (DNA) by DNase II and digesting it with DNase I. The trinucleotide was split into ApA and pTp by DNase I;[4] yet it was also shown that, of the compounds tried, all that were smaller than trinucleotides were resistant to DNase I even though some contained the preferential linkage. Thus ApCp, ApC, TpCp, and TpGp were not hydrolyzed.[4] The presence of mononucleotides[5-7] in the DNase I digest is still unexplained and obviously cannot be accounted for on the assumption that only the pPu-pPy linkage is hydrolyzed.

Recently it was possible to investigate the enzyme streptodornase,[9] that hydrolyzed DNA but not RNA. It was classified as an endonuclease, because it rapidly decreased the viscosity of DNA and because the hydrolysis products were predominantly large oligonucleotides. The digestion mixture was chromatographed on Dowex 1-2X essentially by the method of Sinsheimer[7] with minor modifications.[8] The strength of the eluting buffer was varied in steps

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† The abbreviations used in this article are based on suggestions by Markham, Smith, and Heppel and are now accepted by *The Journal of Biological Chemistry*. Capital letters signify nucleosides: A, adenosine; G, guanosine; T, thymidine; C, cytidine; Pu, unidentified purine; Py, unidentified pyrimidine; and X, Y, and Z, unidentified nucleosides. The letter p *before* a capital letter signifies a phosphoryl group in the 5′ position; *after* the capital letter, in the 3′ position; and *between* 2 capital letters, a secondary phosphoryl group linking the 3′-carbon of the preceding nucleoside with the 5′-carbon of the following nucleoside.

from 0.1 M to 3 M buffer. The tubes obtained with a buffer of a definite strength were pooled and designated as that fraction of a digest (for example, the 2 M fraction for tubes obtained with buffer of 2 M strength).

The simplest way of establishing whether one deals with an enzyme producing 3′- or 5′-terminated products is to isolate mononucleotides and identify them. Since almost no mononucleotide was formed, it was necessary to use a fraction composed mainly of trinucleotides. If a trinucleotide is terminated in 5′-phosphate pXpYpZ → pX + pY + pZ; digestion with venom phosphodiesterase will lead to a mixture of mononucleotides; if the original trinucleotide is terminated in 3′-phosphate XpYpZp → X + pY + pZp, digestion with phosphodiesterase will lead to one third nucleosides, one third nucleotides, and one third nucleoside diphosphate.

Following this reasoning, the 0.75 M fraction from the digest of DNA by streptodornase was digested with phosphodiesterase. Mononucleotides were recovered with 96 per cent yield, and only traces of nucleoside and no nucleoside diphosphate were observed. The original trinucleotide was terminated therefore, in 5′-phosphate.

TABLE 1

CLASSIFICATION OF NUCLEOLYTIC ENZYMES

(1) Susceptible substrates	RNA, DNA
(2) Type of attack	exonuclease, endonuclease
(3) Products	3′-terminated, 5′-terminated
(4) Preferential linkage	Pu ↓ Py, Py ↓ Pu

From the 0.25 M fraction of the streptodornase digest, 2 dinucleotides were isolated and identified as pCpC and pApC. When the 2 M fraction obtained from the digest of DNA by streptodornase was subjected to the action of DNase I, considerable further digestion occurred (FIGURE 1). From the 0.25 M fraction 2 other dinucleotides were identified as pCpA and pTpA; both would be expected on the basis of the specificity of DNase I (TABLE 2).

A summary of the characterization of streptodornase is shown in TABLE 3. The resemblance to DNase I is striking; the only difference appears to be the preference for a reversed sequence of bases adjacent to the susceptible linkage (TABLE 3).

That some DNases produce fragments terminated in 3′-phosphate was first shown by Cunningham *et al.*[1] Shortly thereafter, and independently, Koerner[10] showed that DNase II from spleen produces 3′-terminated mononucleotides. Higher oligonucleotides from the DNase II digest were digested with venom phosphodiesterase. Nucleosides and nucleoside diphosphates were identified among the products.[10-13] Therefore DNase II is characterized as a 3′-former.

DNase II is obviously an endonuclease. The drop in viscosity is very rapid, and the majority of products are oligonucleotides of the size 3 to 10. Surprisingly, however, no definite proof exists for the claim that it has no activity on RNA. The purified preparation of DNase II tested for RNase activity gave

a positive result,[14] and it seems likely that other purified preparations, if tested,[12, 13, 15, 16] would show the presence of RNase activity.*

An attempt was made to characterize the preferential linkage in respect to the sequence of adjacent bases. Five dinucleotides and 1 trinucleotide were

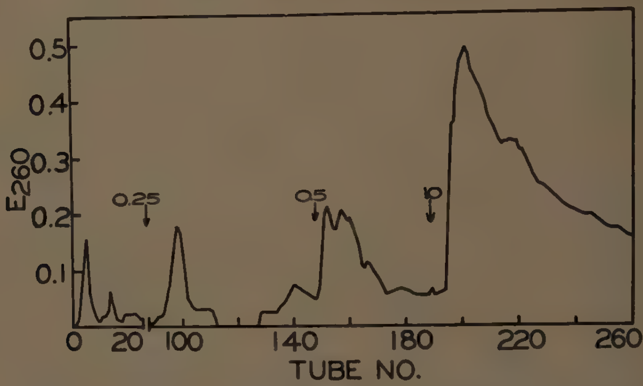

FIGURE 1. Elution pattern of the digest of 2 M streptodornase fraction by DNase I. The 2 M fraction, obtained from a digest of DNA by streptodornase, was lyophilized and dissolved in water. A 20-ml. aliquot containing 480 E_{260} units was adjusted to *p*H 7.5, Mg was added to make a final concentration of 0.025 M, and 300 μg. DNase I was added. The *p*H was maintained at 7.5 by the addition of dilute alkali as required. Total incubation time was 9 hours at 30° C. At this time *p*H was adjusted to 8.5 and the mixture was placed on a Dowex 1-2X column, 15 × 0.9 cm. Elution was done with ammonium formate buffer, *p*H 4.5, with a molarity indicated by the figures above the arrows.

TABLE 2
DINUCLEOTIDES ISOLATED FROM THE 0.25 M FRACTION

Streptodornase	Streptodornase + DNase I
pCpC pApC	pCpA pTpA

TABLE 3
CHARACTERIZATION OF STREPTODORNASE

(1) Susceptible substrates	DNA
(2) Type of attack	endonuclease
(3) Products	5′-terminated
(4) Preferential linkage	pPy-pPu
(5) *p*H optimum	7.0
(6) Metal requirement	Mg^{++} or other divalent cation

isolated from the digest of DNA by DNase II and identified; the combination Pyp-Pup was absent. It was suggested, therefore, that this linkage is preferentially attacked by DNase II.[11]

As an example of a nuclease of different type, I shall consider phosphodi-

* Elsewhere in this monograph, Maver *et al* show the separation of splenic DNase II from RNase using chromatography on substituted celluloses.

esterase, which for a long time has been known to make no discrimination between ribo- and desoxyribo- derivatives but that, only recently, has come to be considered a nuclease. A study of the action of purified phosphodiesterase from venom led to the conclusion that it is an exonuclease.[17, 18] Boman and Kaletta[19] supplied the missing link to this concept when they showed that highly polymerized DNA was attacked by phosphodiesterase. This observation was confirmed with purified phosphodiesterase.[20, 21] When the exonuclease action of phosphodiesterase was first formulated, it was suggested that the degradation of the chain terminating in 5′-phosphate starts from the end carrying the free phosphate,[17] whereas in chains terminating in 3′-phosphate, the attack starts from the end opposite to that carrying the free phosphoryl group.[11] Such an order was deduced from the rate of reaction of different chains.

Singer *et al.*[22] confirmed our postulate that phosphodiesterase is an exonuclease but, contrary to our postulate, showed that in chains terminating in 5′-phosphate the attack begins from the end opposite to that carrying the 5′-phosphoryl group. Razzell and Khorana[23] extended this observation to chains having no terminal phosphate and showed that these compounds are also degraded from the end opposite the free nucleoside. Interestingly, these authors confirmed our previous observation that chains without a free phosphoryl group are hydrolyzed more slowly than those carrying the free 5′-phosphate.

Thus far, no reliable information is available with respect to chains terminating in 3′-phosphate. We have investigated this subject recently with the help of new paper-chromatographic procedure.[24] The method may be summarized as follows: chromatography in the first dimension is made in a mixture of 75 parts of 95 per cent ethanol and 30 parts of 1 M ammonium acetate;[25] in the second dimension, in 80 parts of saturated ammonium sulfate, 18 parts of water, and 2 parts of isopropanol.[26] The method is applicable only to desoxyribo-derivatives. Twelve components—4 nucleosides, 4 nucleotides, and 4 nucleoside diphosphates—are easily separated.

The following experiments were then performed. The 1 M fraction obtained from the digest of DNA by DNase II was used as substrate. It has been shown previously, in Sinsheimer's and our laboratories,[11-13] that the majority of the products formed by DNase II are terminated in 3′-phosphate. Part of the 1 M fraction was rechromatographed on Dowex 1-2X using ammonium formate, *p*H 3.5, for elution. The results are illustrated in FIGURE 2. Peak 5 (FIGURE 2) was lyophilized exhaustively to remove ammonium formate and was subjected to the action of phosphodiesterase. A sample was withdrawn after 10 min., and the remainder was digested for 6½ hours (FIGURE 3). Both samples were chromatographed using the 2-dimensional procedure already described. In the 10-min. chromatogram, nucleosides, some nucleotides, and the bulk of undigested material were detected; nucleoside diphosphates were absent. The experiment would indicate that, in conformity with our postulate,[11] the attack on chains terminating in 3′-phosphate starts from the nucleoside end. The conclusion, however, is valid only if the presence of chains other than those terminating in 3′-phosphate is excluded. An attempt to do so was made by carrying digestion to the end, as evidenced by the disappearance of non-digested material from the chromatograms. The nucleosides and the nucleo-

side diphosphates were eluted, the optical density at 270 mμ was read, and the sum of recovered nucleosides was compared with that of nucleoside diphosphates. Nucleoside diphosphates accounted for about 80 per cent of nucleosides. If one assumes that this value is not an experimental error, it means that only 80 per cent of chains terminated in 3′-phosphate, whereas 20 per cent had no terminal phosphate. If that is the case, no conclusion can be drawn from the early appearance of nucleoside in regard to the starting point of attack by phosphodiesterase. It is conceivable that chains with no terminal can be

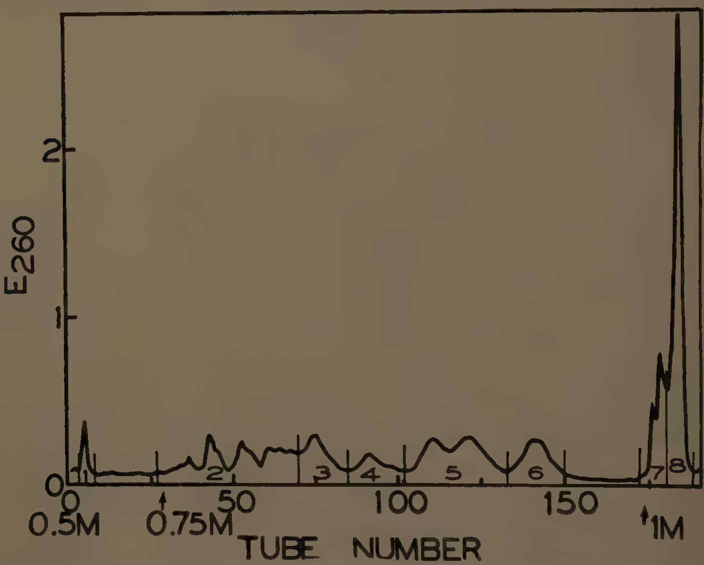

FIGURE 2. Elution pattern of rechromatography of the early part of the 1 M fraction obtained from the digest of DNA by DNase II. DNA was digested with DNase II and was chromatographed on Dowex 1-2X column using ammonium formate buffer, *p*H 4.5, of increasing molarity. 1 M buffer was started after 545 tubes were collected; tubes 560 to 575 were pooled and lyophilized. This fraction was chromatographed again on Dowex 1-2X, using ammonium formate buffer of *p*H 3.5. The pattern obtained in the second chromatography is shown.

completely degraded before the first split of chain terminating in 3′-phosphate will occur.

The other alternative—that the excess of nucleosides has been caused by the 5′-nucleotidase still present in the preparation of phosphodiesterase—has been excluded. The phosphodiesterase has been prepared by a modified method,[21] the summary of which is presented in TABLE 4. The enzyme thus prepared was incubated in a final concentration of 1 U./ml. for 24 hours with the mixture of four 5′-nucleotides at 37° C., *p*H 8.9, in the presence of Mg ion. No inorganic phosphate was found (that is, less than 0.3 μg.), nor were any nucleosides detectable after chromatography in the system previously described.

The third alternative, which is favored at present, is that only 3′-terminated chains result from the action of DNase II. However, during the exhaustive

lyophilization designed to remove formate buffer, some of the terminal phosphates are hydrolyzed off.

Characterization of DNases according to 4 properties listed in TABLE 1 has an additional advantage that has not been mentioned. It offers a possible guide for enzymatic preparation of desired simple substances. If one catalogues enzymes according to the proposed scheme, one can predict a rational

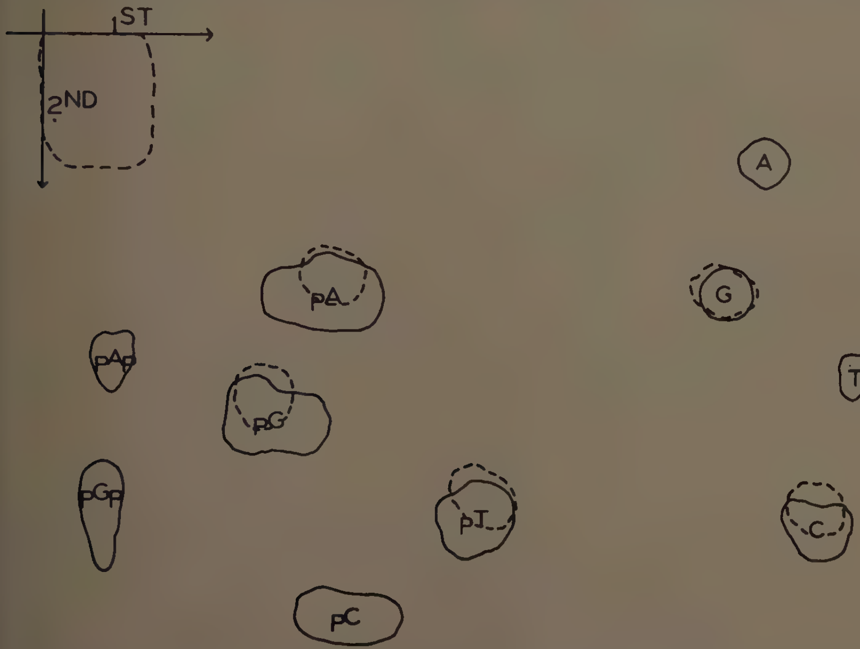

FIGURE 3. Two-dimensional paper chromatography of the digest of Fraction 5 (FIGURE 2) by phosphodiesterase. Fraction 5 was lyophilized, dissolved in 0.5 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer, to which 0.02 ml. of 0.3 M $MgSO_4$ and 0.2 ml. of a solution of phosphodiesterase (equivalent to 0.86 U.) were added. Incubation was at 37° C. A sample of 0.3 ml. was withdrawn after 10 min. and placed on Whatman No. 3-mm. paper. After 1 hour, a second 0.2 ml. of phosphodiesterase was added to the reaction mixture. After 6½ hours the remaining digestion mixture was placed on the paper. The illustration is a composite of 2 chromatograms: the 10-min. sample shown by the dotted line and the 6½-hour sample shown by the continuous line. After 10 min., no nucleoside diphosphates can be visualized, and a considerable part of the material remains close to the origin. After 6½ hours no such material remains, and 2 nucleoside diphosphates are present.

TABLE 4
PURIFICATION OF PHOSPHODIESTERASE

	Potency	Yield percentages
Crude venom	1	100
Acetone, *p*H 4, 46 to 50%	4	30
Ethanol, *p*H 6, 33 to 66%	10	30
CM, 0.2 M acetate, *p*H 6	110	15
DEAE, 0.01 M Tris, *p*H 9	170	6

use of double digestions. Thus, to prepare 5′-terminated mononucleotides, DNase I (or streptodornase) must be followed by venom phosphodiesterase. To obtain 3′-terminated mononucleotides, DNase II must be followed by splenic phosphodiesterase;[28] to obtain mononucleoside diphosphate, the 3′-forming endonuclease must be followed by 5′-forming exonuclease, or vice versa. The preparation of dinucleotides with a desirable sequence may be attempted by using consecutively 2 endonucleases differing only in respect to preferential linkage.

A significant side issue is the usefulness of Ca[bis(*p*-nitrophenyl)phosphate]$_2$ as substrate for phosphodiesterase. Koerner and Sinsheimer[12, 13] and we[14] noticed that DNase II is contaminated with an enzyme (referred to by both groups of workers as phosphodiesterase) capable of hydrolyzing Ca[bis(*p*-nitrophenyl)phosphate]$_2$. This enzyme has been purified about fiftyfold following the activity on synthetic substrate.[27] The purified enzyme had no activity on either 3′- or 5′-terminated chains of desoxyribo-oligonucleotides, or 3′-terminated ribo-oligonucleotides. It had activity on ATP, CTP, and UTP, splitting the terminal phosphate fast, the second phosphate slowly. AMP was totally resistant; the name nucleosidepolyphosphatase is suggested for this enzyme. It is also present in the preparation of splenic phosphodiesterase, according to Heppel and Hilmoe,[28] and can be separated from the enzyme acting on 3′-terminated oligonucleotides by chromatography.[27]

Acknowledgment

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FURTHER STUDIES ON THE UNSTABLE AND "UNNATURAL" DESOXYRIBONUCLEIC ACIDS*

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In the fundamental work of I. R. Lehman and A. Kornberg (see also Bessman *et al.*[1]) on the enzymatic synthesis of desoxyribonucleic acid (DNA), two problems have a connection with the studies my associates and I have been conducting. One of these is related to the utilization of heated DNA as a primer in enzymatic synthesis.

We have been subjecting to heat DNA that has transforming activity, and have found that the loss of biological activity always starts together with the loss of viscosity,[2] even though different genetic markers in the same preparation have different heat stabilities.[3] The important question of whether such heating results in a loss of genetic information could not be answered definitely by these experiments, because a collapsed molecule might conceivably become biologically inactive in the transformation phenomenon and yet retain the information. Another problem is whether the information can *change* upon heating. We have presented evidence[4] that one change that occurs in molecules heated *in vitro* is their unstabilization: the DNA remains biologically active, but loses its stability to heat. On reproduction, the injury is repaired and the molecules become stable again. Thus, the change was not a "mutation *in vitro*." Our attempts to produce a true mutation *in vitro* (a change in DNA that is retained upon reproduction) have not yet led to positive results. The treatments used are heating, ultraviolet irradiation, and mild deamination with HNO_2. Recently, however, we were able to demonstrate a very high mutability and gene unstabilization *in vivo* with *Escherichia coli*,[5] and have advanced a working hypothesis that this is due to the unstabilization of DNA.

Another aspect is the replacement of thymidylic acid by its analogues in the enzymatically synthesized DNA. The problem was to study the replacement of thymine in the DNA of living cells by the thymine analogues and to investigate the biological effects of such replacement.[6-16] In the case of 5-bromouracil in a thymine-requiring strain, about one half of the thymine can be so replaced.[6-8] A substantial part of the thymine can also be replaced by 5-chlorouracil and 5-iodouracil (but not 2-thiothymine, 5-fluorouracil, or uracil). The amount of replacement, however, is smaller than in the case of 5-bromouracil; this amount is correlated with the similarity in size of the halogen substituent and the methyl group.[8] The replacement of thymine by 5-bromouracil is highest in strains requiring thymine for growth;[9] however, the replacement can be achieved in other cases of "disturbances" in DNA synthesis. Such disturbances occur naturally in several mutants (not only thymine-requirers, but also such forms as their "back-mutants"[8] and uracil-requirers)

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or can be produced artificially in wild strains by aminopterin or by high concentrations of 5-bromouracil.[11] This last method, however, was ineffective in introducing into DNA any 5-nitrouracil or 5-methylcytosine. Also, it was not possible by this method to introduce into DNA more thymine than exists normally.[11] Thus, the peculiar base ratios 1:1:1:1 in DNA of *E. coli* could not be changed.

More unexpected was the finding that 5-bromouracil can enter or leave the DNA of apparently nondividing cells, so that the content of 5-bromouracil in DNA at any particular moment seems to be merely a function of the environment.[8, 10] The DNA content per cell remains constant, so there is no obvious synthesis of DNA in these conditions. Our recent study indicates that in the conditions of the experiment there is no "cell-turnover" (death and disintegration of some cells followed by equivalent production of new cells), so that actual "DNA-turnover" is indicated. Such DNA-turnover could occur through the nucleotide exchange; however, the break down and rebuilding of an entire strand of DNA molecule in a nondividing cell is more probable.

The addition of 5-bromouracil to the medium often results in growth inhibition. The only system affected seems to be DNA. Such inhibition is obviously an over-all effect of many possible inhibitory mechanisms.[6] The first mechanism may involve competition between thymine and its analogue for the nucleoside-synthesizing enzyme; indeed, the inhibition can be completely removed by addition of the nucleoside (thymidine). In mammalian tissue, this enzyme synthesizes 5-bromouracil fully half as readily as thymidine.[17] That the desoxyriboside is indeed an intermediate in such a thymine-requiring strain (I) and its back-mutant (II)[12] is indicated by the fact that 5-bromouracil desoxyriboside is as inhibitory as the 5-bromouracil itself.[6] At first, it was not clear why a back-mutant to thymine independence should be inhibited by this analogue of thymidine, although thymidine is not an intermediate in the synthesis of thymidylic acid of a wild strain. It was found[12] that this occurs because the back-mutant is not the wild strain and does not revert to the pathway of synthesis in the latter; instead, the back-mutation creates or brings into action a new pathway in which, indeed, thymidine seems to *be* an intermediate. A similar situation may exist in a back-mutant to uracil independence; the phenomenon may be general and may have significance for evolution.

The second type of inhibition again may be the result of a competition for the phosphorylating enzymes for which 5-bromouracil desoxyriboside is not as good a substrate as thymidine. That the phosphorylated fragments rather than the nucleosides are the precursors has been shown by the studies of T. D. Price *et al.*,[13] which is, of course, in agreement with the mechanism of DNA synthesis of Lehman and Kornberg (elsewhere in this monograph). Their *in vitro* study concerns 1 group of such enzymes, the desoxynucleotide kinases, and they were able to show that these enzymes fail to convert uracil desoxyribotide and 5-methylcytosine desoxyribotide to the triphosphate level.[1] If not supplied as triphosphates, these pyrimidines could not be incorporated into DNA by the enzyme that synthesizes DNA. As mentioned above, in our study *in vivo* these pyrimidines also could not be incorporated. Whether or not 5-bromouracil

desoxyribotide *in vitro* is converted into triphosphate fully as fast as is thymidylate, cannot be said at present.[1]

The third possible cause of inhibition exists if the phosphorylated nucleosides, although formed, compete, but do not fit, the general structure of the DNA molecule, especially as far as the facilities for hydrogen-bond formation and the steric hindrances are concerned. In our study the choice of analogues was such that this possibility was negligible.

The fourth possibility of inhibition exists if DNA that has half of its thymine replaced by 5-bromouracil participates less readily in the activities of the cell. This possibility is perhaps the most profitable one to explore because it would give us information on the structural requirements for DNA serving as heredity determinants. It is conceivable that growing conditions can be established of such nature that the inhibitions due to competitions for enzymes (or, in general, disturbances in DNA synthesis) are not very pronounced; however, the inhibition due to malfunctioning of DNA as heredity determinant should persist.

The experimental results show that there is no correlation between the amount of inhibition and the amount of thymine replacement in DNA;[9, 14] indeed, the analogues or conditions most inhibitory may give no incorporation at all, thus excluding the possibility of malfunctioning of DNA in these cases of inhibition. On the other hand, it is possible to establish growing conditions such that there is maximum thymine replacement, but no growth inhibition and no additional mortality. This indicates that, in the growing conditions in which DNA synthesis is not retarded the malfunctioning of DNA as heredity determinants is not readily demonstrable. An analogous situation was reported recently for the RNA of tobacco mosaic virus:[18] the 5-fluorouracil may partially inhibit the synthesis of viral RNA, but such virus, once synthesized with 5-fluorouracil in its RNA, has an almost unimpaired infectivity.

We have also been interested in the problem of whether such DNA, having about one half of its thymine replaced by 5-bromouracil, can be shown to be in any respect different from the so-called normal DNA. The study *in vitro* has failed to show any differences; the ultraviolet absorption spectrum, the viscosity, and the heat stability were normal.[11]

We turned then to the experiments *in vivo*. We found[15] that cells containing 5-bromouracil in their DNA are as much as 10,000 times more sensitive (on a survival basis) to ultraviolet irradiation than the normal cells and that this sensitization correlates with the extent of thymine replacement by 5-bromouracil in DNA, but not with the inhibition by this analogue. This suggests (although it does not prove) that such DNA, although fully functional, is indeed less stable to ultraviolet irradiation.

As mentioned above, the malfunctioning of DNA containing 5-bromouracil may not be readily demonstrable. Nevertheless, a more thorough search revealed disturbances that persist even after 5-bromouracil has left the DNA during subsequent cell divisions on 5-bromouracil-free medium; obviously, such disturbances cannot be attributed to any inhibition of DNA synthesis by 5-bromouracil.

A search for irregularities produced by 5-bromouracil revealed, first, an increased length of cells (up to 220 μ); these elongated cells can be separated from

the normal ones by centrifugation, and they also can be easily cut to pieces by a micromanipulator. The change persisted after the cells had grown for 60 generations on medium free of 5-bromouracil and, therefore, cannot be considered completely nonhereditary. Further examination of cells grown on 5-bromouracil revealed the occurrence of frequent mutations; some of the mutation rates were higher than 3×10^{-2} per cell per generation. The mutants were characterized by poor growth, but could be made to grow normally on addition of enrichments; this indicates the nutritional character of the mutants. Some of the mutants were unstable for at least 180 generations; a probable cause of this instability may again be the unstabilization of DNA.[16]

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MICROCOCCAL NUCLEASE AND SOME PRODUCTS OF ITS ACTION*

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Some years ago a "desoxyribonuclease" was found[1] in the culture medium of *Staphylococcus aureus* that was unaffected by heating in a boiling-water bath. The enzyme has proved useful in end-group studies of desoxyribonucleic acids and fragments thereof, because the products of its action bear the singly esterified phosphate at the 3′ position.[2, 3]

As was first pointed out by J. D. Smith,[4] ribonucleic acid is also attacked, again only at the 5′ phosphodiester bond, giving rise to nucleoside 3′-phosphates in addition to larger, homologous fragments. Other similarities between the desoxyribonuclease and ribonuclease activities are the unusual stability of the enzyme,[1, 4] the *p*H optimum of 8.6,[1] and the requirement for calcium ion[1, 4] at about 0.01 M concentration.

It appears that, when the enzyme is in the culture medium, it can digest extracellular nucleic acids[5] which, in default of enzyme action, accumulate as part of a slime layer. The enzyme is salted out of the culture medium after boiling and washed with 2.5 per cent trichloroacetic acid saturated with ammonium chloride, followed by 83 per cent ethanol.[1] Since the washing is carried out at room temperature, any traces of relatively labile contaminating enzymes that survived the boiling probably would be destroyed.

Terminal digests of natural nucleic acids are complex mixtures (FIGURE 1). The values, perhaps, match as well as could be expected in view of the initial base differences in the two substrates, except for a marked, unexplained discrepancy between guanylic acid and desoxyguanylic acid.

End-group determinations[2, 6, 7] showed that in several dinucleotide fractions a single sequence accounted for 90 per cent or more of the material. The favored sequences were: desoxyadenylyl desoxycytidylic acid (ApCp) and desoxyadenylyl desoxyguanylic acid (ApGp) in both the desoxyribonucleic acid and ribonucleic acid digests, and desoxythymidylyl desoxycytidylic acid (TpCp) and desoxythymidylyl desoxyguanylic acid (TpGp) in the desoxyribonucleic acid digest; sequence was not determined in other ribodinucleotide fractions. However, both desoxythymidylyl desoxyadenylic acid (TpAp) and desoxyadenylyl desoxythymidylic acid (ApTp) were well represented (about two parts of the former to one of the latter).

There are also trinucleotides or larger fragments that may contain any base of the original substrate. A possible clue to the reason for their presence in the digests is the fact that they are absent after digestion of certain synthetic polynucleotides (the only ones investigated so far) which lack cytosine, uracil, and guanine (FIGURE 2).

The experiment with polyadenylic acid indicated that the sequence ApA must be both susceptible to cleavage (otherwise there would be no digestion)

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and resistant (otherwise there would be no resistant dinucleotide, ApAp). The dinculeotide must be resistant because of size, not because of any particular arrangement of bases. Similarly, it could be shown that some of the mononucleotides and end groups in the desoxyribonucleic acid digest could not have

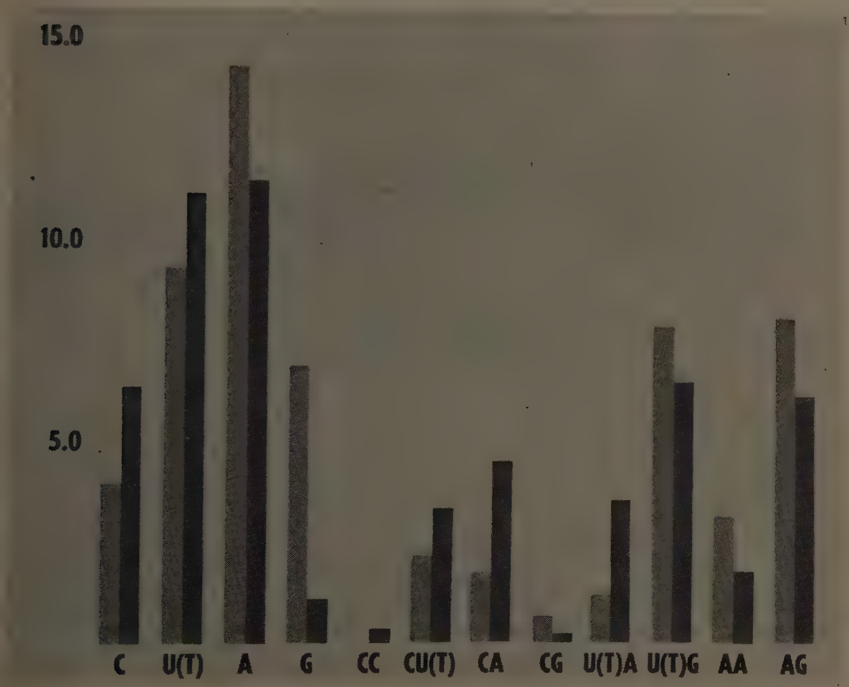

FIGURE 1. Some components of a digest of desoxyribonucleic acid (*dark bars*) and of a digest of a preparation[18] of yeast ribonucleic acid (*light bars*) expressed as percentages of the total material, absorbing at 260 mμ, submitted to ion-exchange chromatography.[19] Completion of enzyme action was judged by cessation of acid production.[1, 3] Thymidylic acid is matched with uridylic acid, U(T), and similarly with the dinucleotides. Cytidylyl cytidylic acid was not detected in the ribonucleic acid digest. The order of letters designating dinucleotide fractions is arbitrary and, in some cases, the fractions were shown[7] to contain both sequential isomers.

In the second of the 2 experiments, with ribonucleic acid, it was found that use of a relatively long column—35 × 1 cm.—obviated rechromatography of dinucleotides, except that AA and UG emerged together.

been formed if all of the dinucleotides identified in the digest represented inherently resistant sequences.

The fortunate characteristic of the enzyme is its specificity for the 5′ phosphodiester bond of desoxyribonucleic acid. In Kornberg's laboratory,[8] incubation with this enzyme, followed by splenic phosphodiesterase, has given the desoxynucleoside 3′-phosphates in 90 per cent or better yield. Nucleosides, representing terminal groups of desoxyribonucleic acid molecules, were also obtained.

The position of the terminal phosphate groups of the products of the enzyme's

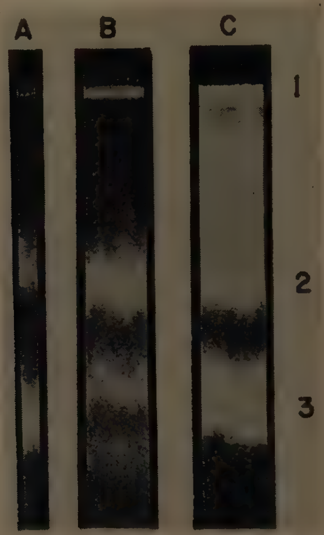

FIGURE 2. Paper strip (Whatman No. 1) chromatograms of digests in isopropanol-concentrated NH_4OH-water (70:10:20),[20] descending. *A* represents polyadenylic acid; *B*, copolymer of thymidylic acid and desoxyadenylic acid;[21] and *C*, ribonucleic acid (for comparison). Digestion was as in the experiments shown in FIGURE 1, except that the concentration of substrate was only 4 mg./ml. for *B*. Similar chromatograms are obtained with both types of nucleic acid, and initial substrate concentrations are not important. The origins are at 1, and comparison with reference spots indicated that dinucleotides are at the level indicated by 2, and mononucleotides (except guanylic acid) at 3. In *B*, the mononucleotide zone is split, because thymidylic acid precedes desoxyadenylic acid. The material between 2 and 1 in *C* is partly dinucleotides containing guanine, which has a retarding effect, and partly (*upper half*) fragments larger than dinucleotides.

TABLE 1

IDENTIFICATION OF NATURAL 3′ ISOMERS OF DESOXYRIBOMONONUCLEOTIDES

Mononucleotide and chromatographic system	R value of 3′ isomer / R value of 5′ isomer	
	Natural 3′ isomer	Synthetic 3′ isomer
Desoxycytidylic, Dowex 1 formate*	0.48	0.48
Thymidylic, *n*-propanol-HCl**	1.1	1.1
Desoxyadenylic, Dowex 1 chloride†	0.53	——††
Desoxyadenylic, $(NH_4)_2SO_4$-isopropanol‡	0.72	——
Desoxyguanylic, 5 per cent KH_2PO_4	0.93‡‡	0.92§

* Dowex 1-X8 formate, eluant 0.01 M formic acid, *p*H 3.0.[11, 12]

** *n*-Propanol-2N HCl (3:1),[13] Whatman No. 1, descending.

† Dowex 1-X8 chloride, eluant 0.002 M HCl, *p*H 2.8.[14]

†† A ratio of 0.59 was found for synthetic isomers in a similar system.[15]

‡ Eighty per cent saturated $(NH_4)_2SO_4$-isopropanol (98:2), Whatman No. 1, descending.[16]

‡‡ Whatman No. 1 cut to special shape,[3] ascending.

§ Hayes *et al.*,[15] using a layer of isoamyl alcohol.[17]

action was first indicated by the resistance of the mononucleotides to 5′ nucleotidase[1] and by the slow release of a nucleoside 3′,5′-diphosphate and a nucleoside from 1 of the dinucleotides when it was incubated with snake venom phosphodiesterase.[2] Further, the mononucleotides were identified chromatographically (TABLE 1)[3] in systems capable of separating nucleoside 3′-phosphates from 5′-phosphates.

The isomers of desoxycytidylic acid could also be differentiated by their ultraviolet spectra at *p*H 3.

Desoxynucleoside 3′-phosphates show less affinity for ion-exchange resins than do the corresponding ribomononucleotides and can be separated from them, whereas the pairs of 5′ mononucleotides move together. It was not possible to dephosphorylate the 3′ desoxymononucleotides specifically with 3′ nucleotidase[9] or cerium hydroxide.[10] The rye grass 3′ nucleotidase, accordingly, appears to be specific for ribose compounds.

Acknowledgments

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MAMMALIAN ENZYMES OF DESOXYRIBONUCLEIC ACID SYNTHESIS*

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A discussion of desoxypolynucleotide and desoxyribonucleic acid (DNA) synthesis should include something about what animal enzymes can do in this respect; what bacterial enzymes and our more ingenious chemists can do, does

TABLE 1

REQUIREMENTS OF CALF THYMUS POLYMERASE*

Reaction mixture	H^3-thymidine triphosphate incorporated, $\mu\mu$moles
Complete	100
Omit dATP	9
Omit dGTP	13
Omit dCTP	28
Omit dATP, dGTP, dCTP	2
Omit Mg^{++}	0
Omit DNA	0

* Complete system contained, in 0.2 ml.: 5 mμmole of each desoxynucleoside triphosphate (H^3-thymidine triphosphate containing 5.2×10^6 cpm/μmole); 25 μg. DNA; 195 μg. enzyme protein; 2 μmole Mg^{++}; and 5 μmoles glycine buffer, pH 9.0. Incubation: 20 min. at 37.5° C.

TABLE 2

TISSUE DISTRIBUTION: SOLUBLE DESOXYNUCLEOTIDE-POLYMERIZING ENZYMES[5]

Tissue*	Relative activity†	Tissue	Relative activity
Thymus	100	Lung	4
Small intestine	30	Brain	3
Regenerating liver	28	Heart	3
Spleen	22	Pancreas	1
Testes	9	Skeletal muscle	1
Kidney	8	Flexner-Jobling carcinoma	130
Liver	7	Walker 256 carcinoma	300

* All from 140- to 160-gm. male albino rats.

† Relative activity is specific activity of enzyme compared to thymus as 100 per cent.

not provide a balanced picture. For that reason, I propose to make a case for other polymerizing enzymes, particularly calf thymus polymerase, an enzyme that has been investigated in two laboratories[1, 2] The requirements of a purified calf thymus polymerase are presented in TABLE 1.

Considerable interest surrounds desoxynucleotide-polymerizing enzymes, such as the *Escherichia coli* enzyme.[3] What is the best source for such enzymes? Despite the fact that bacteria are a concentrated source of the enzyme, for

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many investigators, it is more feasible to obtain 100 kg. of calf thymus than 1 kg. of *E. coli*; logistically speaking, therefore, calf thymus is an important source of polymerase. It seems worthwhile to attempt purification of thymus polymerase for that reason alone.

In addition, there are other facets to consider. From Laskowski's review,[4] an informed biochemist can no longer call an enzyme a nuclease or a phosphodiesterase and really convey a complete meaning. One must at least specify spleen diesterase and pancreatic DNase. There are different nucleases and different diesterases. Who is willing to say that there is only one type of polymerase? Desoxypolynucleotide-polmerizing enzymes are ubiquitous even in mammalian cells (TABLE 2); who would be so atavistic as to say they are all the same?

The problem of nuclease and polymerase may be interdigitated, that is, some nuclease may be necessary for polymerase action. It is possible, however, to make extracts of mammalian cells that do not contain appreciable amounts of desoxyribonuclease and do contain polymerizing activity. Here again, thymus is of interest in that its principal depolymerizing activity is an acid desoxyribonuclease.[6] Ultimately, therefore, it may provide a polymerase that is free of desoxyribonuclease activity. This would be of some importance in investigations of polymerization mechanism. Purification schemes[7, 8] already devised for the thymus desoxyribonuclease provide landmarks for fractionations designed to avoid it.

Polymerase now has been purified about thirtyfold from soluble fraction of fresh calf thymus. Further purification and detailed investigation of the polymerization mechanism will be required in order to answer the questions raised above.

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THE STATE OF DNA IN THE RESTING-CELL NUCLEUS

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In connection with the general topic of enzymes hydrolizing desoxyribonucleic acid (DNA), I shall review briefly some information concerning the condition of DNA in the resting-cell nucleus and the effect of desoxyribonuclease (DNase) action on DNA as it exists *in situ*. This topic has some bearing on the interpretation of results obtained with various methods for isolating DNA, on cytochemical separations in general, and on the structure of chromosomes and the maintenance of correct gene sequence. Finally, it may possibly have bearing on the process of replication of DNA in the cell or in the test tube, and on the function of DNA in the resting cell.

In the past it was assumed universally that the DNA of the nucleus of the resting mammalian cell existed solely in the form of a salt with histone, with no covalent attachment to any protein of the nucleus; this view is still held by many. It is possible, in fact, to extract DNA, together with histone, from certain types of whole tissue and from some types of isolated nuclei by the use of 1 to 2 M saline solutions at neutral *p*H, and the DNA thus extracted evidently is present in the solution as a histone-DNA salt-type complex. Moreover, such DNA-histone when freshly isolated appears to differ in physicochemical properties from reconstituted DNA-histone complexes.

However, the finding that a certain situation prevails in aqueous extracts of a tissue is not evidence that this situation existed in the cells of that tissue before they were broken and subjected to the extracting medium, unless it can be demonstrated that autolytic changes have not set in as the result of the liberation or activation of autolytic enzymes during the extraction procedure.

It was demonstrated in our laboratory that nuclei isolated from liver cells under certain conditions would form strongly recoilable gels* at very high dilution upon the addition of dilute alkali or molar saline at *p*H 7.0, whereas nuclei isolated under other conditions would not do so.[1] DNA was easily extractable from the latter nuclei as a histone salt, but was not easily extractable from the former. It was shown that small amounts of DNase I or II, as well as protease, would cause the DNA of the gelable nuclei to become easily extractable, and it was demonstrated in our laboratory[2] and in at least one other[3] that DNase I of liver cells can bring about a loss of gelability of the liver cell nuclei. My associates and I also demonstrated that if mitochondria were kept intact during the procedure for isolating nuclei, gelable nuclei were obtained even though nothing was added to inhibit DNase action.[2] The importance of maintaining mitochondrial (or lysosomal) integrity during isolation of cell nuclei in order to prevent degradation of the DNA nucleoprotein by DNase action has been emphasized by Rotherham *et al.*[4]

* The gels in question are easily distinguishable from gels formed by Na-DNA itself, owing to their high degree of recoil after rapid stirring and to their persistence at considerably higher dilutions than are required to break Na-DNA gels.

These results lead to the reasonable conclusion that there is firm binding of the nuclear DNA to some nuclear constituent, and that gelability of the nuclei can be used as a criterion of this firm binding. It was found by Mirsky and Ris[5] that an apparently firm binding of at least some DNA to the residual nonhistone protein of chromosomes does in fact occur; and in our laboratory it has also been shown[6] that the residual, nonhistone protein is the material to which the DNA is firmly bound. This was done both by demonstrating that the removal of lipid of histone would not destroy the gelability of isolated liver cell nuclei, and by studying the DNA-residual protein complex through enzymatic degradation followed by electrophoresis on paper.

Much thought and effort has been spent in attempting to understand the molecular nature of isolated DNA, and this has culminated in the two-stranded model of Watson and Crick, which seems to be compatible with all of the physicochemical and physical investigations of DNA to date. However, it appears that no really satisfactory way has yet been proposed to make the Watson-Crick model account for the dynamics of the cellular duplication of DNA molecules. It seems possible that it will be necessary to take into account the mode of binding of DNA to protein in the chromosomes in order to understand this process completely, as well as to understand the replication of chromosomal structure. Even in attempting to explain the metabolic function of DNA in the resting cell, which may be concerned with the production of specific ribonucleic acid (RNA) molecules, it is possible that a knowledge of the way DNA is bound to nonhistone chromosomal protein will be required. However, the phenomenon most urgently requiring an understanding of the DNA-residual protein complex is that of *the maintenance of a fixed gene sequence in the chromosome.*

There seem to be three general possibilities for explaining gene sequence in chemical terms. One possibility is that there is a longitudinal protein structure with DNA occurring as branches; a second is that DNA fibers alternate with protein fibers throughout the length of the chromosomal fibrils (chromonemata); and a third is that the DNA molecule extends throughout the length of the chromosome, and that molecular interruptions occur at points where one gene is separated from another. These three possibilities are illustrated in FIGURE 1.

At least one model of chromosomal structure has already been proposed that is an elaboration of the first general type given above.[7] This model, recently published by Taylor, consists of two strands of protein (chromatids) that extend the length of the chromosome and bear lateral DNA branches. The model would fit in well with the concept of the DNA-residual protein complex, and would explain the maintenance of a fixed gene sequence. A fairly convincing electron micrograph was offered in support of the model.[7]

Other arguments can be offered in favor of the first general type of model presented above. One is that a model of this type would lend itself to an explanation of the ability of chromosomes to fuse their parts after being broken, without the production of gross genetic damage. If the continuity of the protein backbone of a chromosome is not involved in genetic specificity, the repairing of breaks in the peptide chains should not cause appreciable genetic

difficulty, whereas the breaking and subsequent repair of DNA chains might well be expected to cause considerable genetic effects, if DNA itself is the genetic material, as now seems certain.

A second argument in favor of the first type of model is that the residual chromosome of Mirsky, from which most of the DNA has been extracted,

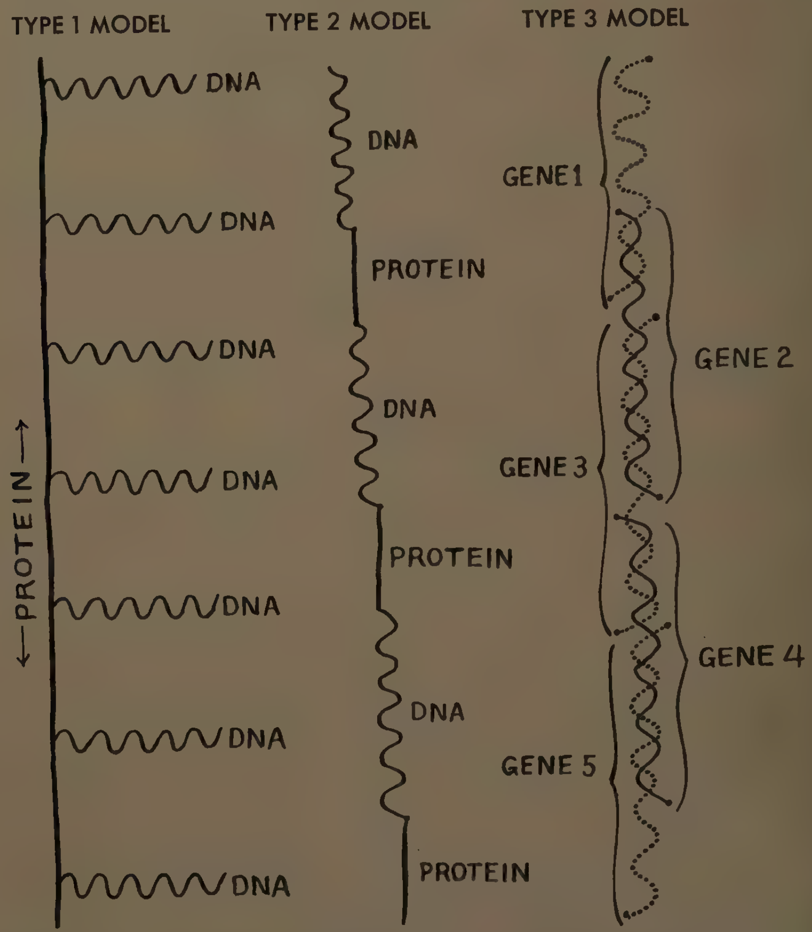

FIGURE 1. Three possible chromosomal models. No commitment is implied as to what sort of DNA structure may be found in the Type 1 or Type 2 models.

retains the general form of the chromosome.[8] Some investigators may feel that this argument is not conclusive, however, since a small percentage of DNA is generally still present in the residual chromosome, together with some RNA and the nonhistone residual protein.

A third argument in favor of the first type of model, although not contrary to the second type, is that, in the past, it has been stated that trypsin in the presence of buffer can destroy the longitudinal continuity of the giant chromo-

somes of the salivary glands of *Drosophila* larvae.[9,10] Argument concerning the rôle of the buffer[11] does not appear to invalidate this evidence. However, it has been found by Gall[12] and by Callan and Macgregor[13] that the longitudinal continuity of oöcyte lampbrush chromosomes is not destroyed by trypsin under conditions where the loops are rapidly digested. Moreover, it is claimed by Callan and Macgregor[13] that the longitudinal continuity of the lampbrush chromosome *is* destroyed by DNase. Taken together, these last 2 observations would seem at first sight definitely to exclude the Type 1 model, at least in the case of the lampbrush chromosomes, leaving Types 2 and 3 for further consideration.

Of the latter 2 types, Type 3 seems the least plausible. It is possible to imagine the entire gene complement of a chromosome as spread longitudinally along a number of like DNA fibers of the Watson-Crick type, which run the entire length of the chromosome, if one assumes the presence of staggered interruptions at points marking the end of one gene and the beginning of another (FIGURE 1, Type 3 model). In such a system, only 1 strand of the double helix would be functioning genetically, but this system would destroy the complementarity of the two helix fibers and would not lend itself easily to an explanation for replication of the DNA structure. Furthermore, it is doubtful whether a DNA molecule of the Watson-Crick variety, having a molecular weight of about 6×10^6 and consisting of about 9700 Watson-Crick base pairs, would be long enough to reach from one end of an uncoiled chromosome to the other, although the length might be of the right order of magnitude.

If one considers a fourth type of chromosomal model, namely one consisting of uninterrupted DNA double helices running the length of the chromosomes, a problem that arises is that of how the various genic functions can be separated. This problem might be solved by the use of the commaless coding scheme of Crick and Orgel.[17] However, there remains the problem of how to fit the residual protein into such a scheme.

It may be noted in passing that it is very difficult to reconcile the molecular weight of the DNA particle (about 6×10^6) with single-gene activity. Genes must operate, directly or indirectly, to position the amino acids of peptide chains in the proper sequence, and it is difficult to understand how a DNA fiber consisting of about 9700 Watson-Crick paired nucleotides, with a fiber length of about 3.3 μ, could transmit information in such a way as to position the amino acids correctly in a peptide chain containing perhaps 100 amino acid residues with a chain length of only about one one-hundredth that of the DNA chain. According to any of the schemes for information transfer thus far proposed,[14-16] it would seem that the size of the DNA molecule would be too great to correspond to the function of a single gene, unless by far the greater part of the molecule were not being used.

Considering as a whole the evidence presented above, it would seem that the bulk of the material favors a chromosomal model of Type 1, with the possible exception of the lampbrush chromosome. The latter might constitute a special case; or it might be that the results of Callan and Macgregor,[13] which were concerned with the destruction of chromosomal continuity through DNase action, depend upon some kind of autolysis following the action of the DNase or, possibly, upon contamination of the DNase with a very small amount of

protease, the action of which becomes apparent only after the prior action of the DNase. The Type 2 model cannot be entirely excluded, however, on the basis of the evidence presented, but the Type 3 model seems improbable. The Type 4 model as it stands seems difficult to reconcile with work on mammalian chromosomes, but it may be possible to combine it with the Type 2 model to give a hybrid fifth type.

Whether we accept a model of Type 1 or of Type 2, it seems that the blocking of one or both ends of the DNA chain by an attachment to protein may have an important bearing on the duplication of DNA molecules and on the functioning of DNA in the resting cell, where possibly it serves as a template for RNA synthesis. It would seem, for this reason as well as for the other reasons already mentioned, that the nucleoprotein gels referred to above, which appear to consist of DNA rather firmly bound to nonhistone protein, are worthy of considerably more attention than they have been given thus far.

In this discussion, no consideration has been given to the role of the histone, which of course must be of considerable importance. The nucleoprotein of Doty *et al.*[18] doubtless consists of histone wrapped around a DNA double helix in some manner. My disagreement with Doty[19] concerning DNA-nucleoprotein seems to be largely resolved except in regard to the interpretation of gelation. Resolution of the disagreement has amounted to a recognition by both Doty and me that two types of nucleoprotein exist—one a DNA-histone (Doty's nucleoprotein) and one the DNA-residual protein of mine and of other investigators.

In regard to the role of histone, I propose what I believe to be a new idea, namely that histone may serve to block one of the two strands of the DNA double helix. One of the strands is redundant as far as information storage is concerned and, therefore, it may be necessary to block possible template function of one strand of DNA in whatever process may exist for the expression of gene action in RNA or protein synthesis. The mode of attachment of DNA to the residual protein could determine which chain of the DNA was to be blocked by the histone.

In any case, whatever the function of the histone may be, it is necessary to include this material as molecular wrapping of some sort for the strands in order to complete the chromosomal models presented in this paper.

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ON THE SPECIFICITY OF THE SNAKE VENOM PHOSPHODIESTERASE

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The last two years have led to a great increase in our knowledge of the specificity of the venom phosphodiesterase; the present situation is reviewed elsewhere in this monograph by Hilmoe,[1] Laskowski,[2] and Tener.[3] The purpose of this article is to describe and discuss a few kinetic experiments with diesterase degradation of desoxyribonucleic acid (DNA) in the presence of different metals and a chelating agent. The initial idea behind these experiments was that the formation constants for the complexes between divalent cations and the phosphates of a polynucleotide chain may vary because of the surrounding nucleotides. If so, a careful selection and control of the cation concentration might offer possibilities for a limited but specific digestion. The results obtained thus far do not seem to contradict this working hypothesis, but at the same time they indicate that the description of the specificity of the diesterase has not yet been completed.

The starting material for the enzyme preparation was freeze-dried venom from ringhals cobra* (*Hemachatus haemachates*, formerly named *Sepedon haemachates*). Our work with different venoms[4, 5] indicates that an evident autodigestion occurs during the "ordinary" collection procedure, and this fact may be the explanation for the difficulties many workers have experienced in reproducing enzyme preparations from snake venom. The freeze-dried ringhals venom, however, has proved to be reproducible; it is hoped to achieve a convenient and satisfactory preparation procedure for diesterase from this material. The sample used in the following experiments was purified by chromatography on DEAE cellulose.[5] The enzyme was not definitely free from contaminating 5-nucleotidase, but the latter enzyme can hardly interfere with the hydrolysis of DNA when this is followed by the increase in the extinction of the lanthanum acid–soluble nucleotide material.[4]

The DNA used was from one preparation of calf thymus prepared according to Kay *et al.*[6] The substrate solution was 0.1 per cent DNA in 0.1 M "Bis"-HCl buffer at a *p*H of 9.1.† Before the experiments, both enzyme and substrate were adjusted to 0.001 M concentration of ethylenediamine tetraacetic acid (EDTA) and, afterward, to the different concentrations used of calcium and magnesium. After 20 min. of preincubation at 37° C., 100 μl. of enzyme (with E_{280} = 0.23) was added to 1 ml. of DNA solution. At different times aliquots of 100 μl. were removed and added to cold samples of 1 ml. of 0.01 M $La(NO_3)_3$ in 0.1 M HCl. At the end of the experiment, these solutions were centrifuged and the extinction at 260 mμ of the supernatant solution was measured. FIGURE 1 shows the results from 4 such experiments made with the

* Obtained through the cooperation of J. H. Mason, The South African Institute for Medical Research, Johannesburg, Union of South Africa.

† "Bis" = bis(hydroxymethyl)methylaminomethane, product of Commercial Solvents Co., New York, N. Y.

same enzyme and substrate solutions adjusted to different concentrations of EDTA, calcium, and magnesium, as given in the figure.

I am very conscious of the complexity of the system studied, and that it may be very difficult to make correct interpretations without much more work, especially identification of some products of the reaction. On the other hand, I feel that a monograph of this kind may be the proper vehicle for a critical

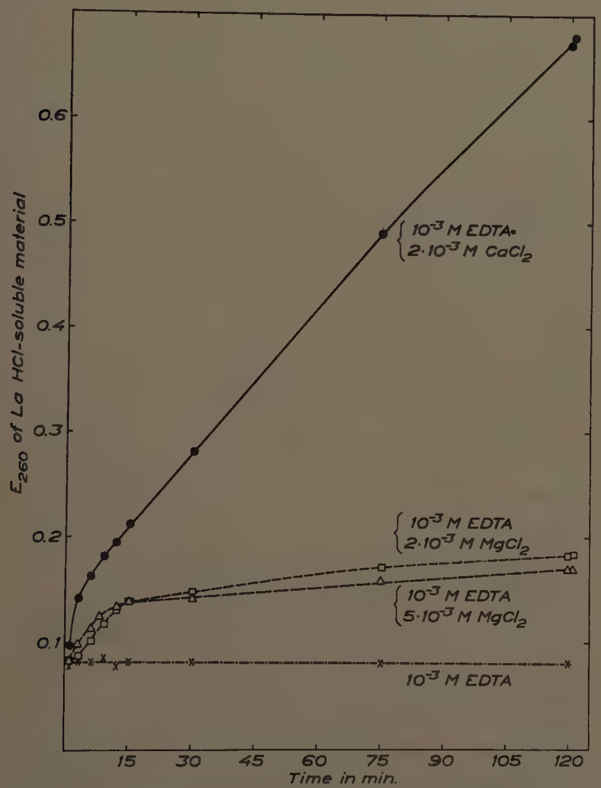

FIGURE 1. The liberation of lanthanum acid–soluble nucleotide material from DNA incubated with snake venom phosphodiesterase in the presence of 10^{-3} M EDTA (*crosses*); 10^{-3} M EDTA and $2 \cdot 10^{-3}$ M $CaCl_2$ (*closed circles*); 10^{-3} M EDTA and $2 \cdot 10^{-3}$ M $MgCl_2$ (*open squares*); and 10^{-3} M EDTA and $5 \cdot 10^{-3}$ M $MgCl_2$ (*open triangles*).

discussion of some of the possible explanations that can be given for these results.

When DNA and venom diesterase are incubated with no additions, the time-activity curves may vary from one preparation to another, probably reflecting the uncontrolled metal concentrations in both the enzyme and the substrate. The straight line (with crosses) in FIGURE 1 shows that EDTA alone completely inhibits the enzyme and that the reaction thus requires a metal. It may also be concluded from FIGURE 1 that the formation constants for the active metal complex must be lower than the constants for the formation of the Mg-EDTA

or the Ca-EDTA complexes ($5 \cdot 10^8$ and $4 \cdot 10^{10}$, respectively.)[7] In a purely descriptive way, it may be stated that, with an excess of calcium the reaction proceeds almost to completeness (*closed circles*), while an excess of magnesium (*open triangles and squares*), after 15 to 20 min. will decrease the reaction rate to nearly zero. It appears reasonable to interpret the shape of the calcium curve (*closed circles*) as a result of the fact that 2 reactions are recorded, 1 dominating the first 10 to 15 min., the other responsible for the remaining or linear reaction. This interpretation may be supported by some kinetic experiments with the degradation of labeled DNA, recently described by Adler *et al.*,[8] who concluded that venom diesterase showed a higher affinity for some parts near the end of the intact DNA molecule than for the rest of the polynucleotide chain.

Snake venoms are known to contain enzymes that hydrolyze adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN).[9] When assayed, our diesterase preparations have always been found to contain an activity toward ATP corresponding to the diesterase concentration. I have recently determined that my best preparations of diesterase, from both ringhals and rattlesnake venom, split ATP and DPN, while adenosine diphosphate (ADP) is only slowly attacked. In the case of ATP, the main reaction products formed were identified (with paper chromatography) as adenosine monophosphate (AMP) and pyrophosphate. With DPN, two nucleotides were obtained and one was identified as AMP. Neither of the two reactions seem to go to completeness. With respect to these activities, the enzyme preparation thus resembles the nucleotide pyrophosphatase of Kornberg.[10]

The work of Razzell and Khorana[11] has shown that venom diesterase degrades a synthetic polynucleotide stepwise, starting from that end of the chain that has a nucleotide with a 5′-phosphate esterified to the 3′-hydroxyl of the next residue. Thus, if the same enzyme hydrolyzes the polynucleotides ATP and DPN, it would imply that the active site had an affinity for the diesterified phosphate of the 5′-nucleotide residue present in the "end" of these molecules.

The present experimental material may not be regarded as proof that venom diesterase can split certain pyrophosphate bonds, but probably some enzyme preparations used in the study of DNA have had this ability. Consequently, the possibility exists that DNA, in addition to the 3′,5′-phosphodiester bonds, may contain *small* amounts of pyrophosphate bonds. If so, this could account also for some 3′,5′-diphosphate nucleotides.[12, 13]

Smith and Alberty[14] have shown that the stability constants for magnesium complexes with pyrophosphate nucleotides are greater than those for the complexes with calcium. It is thus possible that, in FIGURE 1, the reactions with an excess of magnesium (where no free calcium can exist) involve the hydrolysis of a few pyrophosphate bonds, and that the calcium curve (where the magnesium of the DNA and the enzyme preparations is available) is made up from both the splitting of pyrophosphate bonds and the stepwise degradation of the polynucleotide chain.

This paper contains several speculations that may be justified only when coming from a discussant. However, one fact appears unescapable; namely, that it is better to try to control the metal ion concentration rather than to utilize the metals that happen to be left in the preparations.

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